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Effects of Gibberellin A_3 on the Chemical Composition of Celery Plants[a]

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(Manuscript received April 22, 1960)

SUMMARY

Detailed studies of the composition of young celery plants treated with gibberellin revealed changes in the organic and inorganic composition of tops or roots associated with increases in yield of dry matter. Non-reducing sugars and polysaccharides were increased in the tops, and nitrate nitrogen reduced, whereas non-reducing sugars were increased and polysaccharides decreased in the roots. The most significant differences in mineral composition attributed to gibberellin treatment were an increase in calcium, and decreases in boron, phosphorus, and copper in the tops.

The effect of gibberellin on the chemical composition of plants has been the subject of several investigations. Brian *et al.* (1954) found an increase in total dry weight and soluble carbohydrates in peas and wheat plants treated with gibberellin. Hayashi *et al.* (1953) found in rice an increase in dry weight, hemi-cellulose, and cellulose, and a decrease in sucrose and starch; the water-soluble pectic substances and gums remained unchanged. Yabuta *et al.* (1951) found in rice seedlings no significant effect on moisture, ash, and total nitrogen contents, but a decrease in total sugars although the content of reducing sugars was similar to that in controls. The percentage of dry matter and the mineral content of grass, according to Wittwer *et al.* (1957), did not differ significantly between gibberellin-treated and untreated plants. Yabuta *et al.* (1943), Burk and Tso (1958), and Parups (1959) reported that foliar applications of gibberellin decreased the nicotine content of tobacco plants. Morgan and Mees (1956) observed an increase in crude protein in gibberellin-treated grass plants, and no change in phosphorus, potassium, sodium, calcium, barium, magnesium, copper, aluminum, and tin.

Celery plants are very responsive to foliar sprays of gibberellin, often showing within 2–3 weeks a 25–40% increase in weight of stalk and length of petiole (Wittwer and Bukovac, 1958). These phenomenal effects on growth and yield led to inquiries as to possible changes in chemical composition as related to nutritive value. Accordingly, the comparative contents of organic and inorganic constituents were determined in gibberellin-treated and untreated plants.

MATERIALS AND METHODS

Celery plants (var. Utah 10-B) were started in sand in a greenhouse, and when 3–4 true leaves had developed the plants were transferred to aerated solution cultures containing half-strength Hoagland and Arnon (1950) nutrient solution. Plants were grown during the short days (9–11 hours) of early spring and at a night temperature of 10–15°C. Ten microliters of an aqueous solution containing 10 μg of gibberellin A_3 were delivered from a pipette to the youngest unfolding leaf when 5–6 true leaves had appeared. Two replications of 6 plants each of both treated and untreated plants were used. When the young plants had formed 10–12 true leaves (15 days after treatment) the plants were harvested, separated into tops and roots, dried immediately at 70°C in a forced-air oven, and prepared for chemical analyses by grinding in a Wiley mill to pass a 60-mesh sieve.

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Carbohydrates and ether extract in the tops were determined by a procedure described by Sell *et al.* (1946). Nitrogen determination was by a semimicro-Kjeldahl method, using potassium sulfate and copper sulfate as a catalyst. Ammonium, amide, nitrite, and nitrate nitrogen were determined by the method described by Varner *et al.* (1953). In the roots the ether extract, nitrogen fractions, and nitrogen were determined as in the tops. Because of the limited amount of root material, the carbohydrates were analyzed by the micromethod described by Somogyi (Association of Official Agricultural Chemists, 1955). Standard glucose solutions were employed to develop technique and to check results. Mineral analyses were determined by the A.O.A.C. spectrographic method wherein aliquots of the ash were placed on flat polished electrodes instead of graphite-filled crater electrodes.

RESULTS AND DISCUSSION

The data for organic constituents are summarized in Table 1. Accumulation of dry matter in the tops was greater in the gibberellin-treated plants than in the controls. Differences in the roots were not significant. Non-reducing sugar was found in larger quantities in both the tops and roots of the treated plants, but there was less reducing sugar in the roots. Polysaccharides were higher in the treated tops but lower in the roots. Differences in the ether extract, starch, Kjeldahl nitrogen, ammonium nitrogen, amide nitrogen, or nitrite nitrogen were not significant in either tops or roots. The percentages of nitrate nitrogen in the tops and roots were much less in the treated plants than in the controls.

Table 1. The effects of gibberellin A_3 on the total dry weight, organic constituents, and certain nitrogen fractions of celery plants (results expressed as per cent dry weight).[a]

	Tops		Roots	
	Control	Treated	Control	Treated
Dry weight	17.50	22.83	2.97	2.85
Ether extract	3.84	3.51	2.25	2.24
Non-reducing sugar	2.39	3.67	0.09	1.66
Reducing sugar	1.61	1.62	0.75	0.53
Starch	0.88	0.94	trace	trace
Polysaccharides[b]	1.36	1.80	4.20	3.18
Kjeldahl nitrogen	5.63	5.45	4.32	4.51
Ammonium nitrogen	0.16	0.16	0.17	0.14
Amide nitrogen	0.08	0.10	0.07	0.06
Nitrite nitrogen	0.15	0.16	0.35	0.26
Nitrate nitrogen	0.71	0.14	0.29	0.17

[a] Each value is a mean of single determinations on 2 replicate samples.
[b] Acid-hydrolyzable polysaccharides other than starch.

Table 2 gives the percentages of 8 different inorganic elements in the gibberellin-treated and untreated plants. Treated plants contained slightly less iron, manganese, and phosphorus, and a marked decrease in boron and copper, but more calcium than the controls. There were no significant differences in magnesium and zinc.

Table 2. The effect of gibberellin A_3 on the inorganic constituents of celery plants (results expressed as per cent dry weight).[a]

	Tops	
	Control	Treated
Boron	.0060	.0025
Calcium	2.05	2.79
Copper	.0062	.0036
Iron	.0089	.0075
Magnesium	.21	.21
Manganese	.0046	.0041
Phosphorus	.64	.54
Zinc	.0041	.0041

[a] Averages of replicated spectrographic determinations.

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Production of Irradiation Odors from Beef Protein Fractions and Their Derivatives

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(Manuscript received May 18, 1960)

SUMMARY

This report contains information on some odors produced by gamma irradiation of several beef protein fractions and their derivatives. The odor was shown to vary with protein class, with molecular weight and electric charge, with the medium, and with the availability of functional groups. The unorthodox physical and chemical responses of an odor characterized as a "wet dog" odor are contrasted with those of a "wet chicken feather" odor. Of chief significance is the large variety of irradiated odors that can be produced from a single starting material as conditions are varied.

Since 1954 the Quartermaster Corps of the United States Army has been engaged in an investigation to determine the feasibility of preserving ration items by gamma irradiation. With beef, undesirable odors and flavors are produced that decrease acceptance. Some of these odors have been identified by various workers, but there is no general agreement on the relative importance of each of these odors. Among the odor compounds identified are hydrogen sulfide and the low-molecular-weight mercaptans (Batzer *et al.*, 1959), several amines (Burks *et al.*, 1959), dimethyl sulfide and dimethyl disulfide (Merritt *et al.*, 1959), and 3-methylmercaptobutyraldehyde (methional) (Oro *et al.*, 1959; Patton and Barnes, 1958; Witting and Batzer, 1957). There is no exclusive source from which the odors are produced. That protein and/or low-molecular-weight nitrogen-containing compounds are implicated can be seen from an appraisal of the various odors identified (Batzer *et al.*, 1959; Burks *et al.*, 1959; Merritt *et al.*, 1959). Since the radiolability of polysaccharides (Schoenherd *et al.*, 1949) and of animal fats (Hannan and Shephard, 1954) is also recognized, it is apparent that all of the components of beef contribute in some degree to the irradiated odor.

In a previous study in this laboratory, a water-soluble protein-containing fraction, obtained by refluxing beef with water, produced upon irradiation an odor very similar to that produced from irradiated cooked beef (Hedin *et al.*, 1960). The odor appeared to be associated with sulfhydryl or closely related compounds since the contents of cysteine and methionine were decreased and the odor could be quenched by sulfhydryl reagents. This report contains additional information on the odors produced by gamma irradiation of several beef protein fractions and their derivatives. Also discussed is the production of odors from undenatured beef protein fractions.

EXPERIMENTAL

Preparation of protein fractions from beef.

a) Twelve hundred grams of defatted beef chuck was diced and refluxed overnight with 1200 ml of water. The extract was collected by filtration, concentrated to 250 ml under reduced pressure, and dialyzed at 4°C against water for several days with periodic changes. The nondialyzable fraction was dried from the frozen state (Hedin *et al.*, 1960). Yield: 28.0 g.

b) Extracts were also prepared by stirring the same quantity of diced beef in cold water and in

[a] This paper reports research conducted by the Quartermaster Food and Container Institute for the Armed Forces and has been assigned No. 2009 in the series of papers approved for publication. The views or conclusions are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

10% sodium chloride solution. Dialysis was at 4°C without prior concentration, and the non-dialyzables were dried from the frozen state. Yield from water extract: 28.3 g. Yield from saline extract: 36.4 g.

c) Ammonium sulfate fractionation. Water was added to each of the freeze-dehydrated preparations to form 2% suspensions. Ammonium sulfate was then added to make the solution 25% saturated (706 g/L at 4°C constitutes a saturated ammonium sulfate solution). Similar adjustments were made to give 50%, 75%, and 100% saturated solutions. Precipitates were collected by centrifugation and dialyzed with water before being lyophilized. The filtrate from the 100% saturated ammonium sulfate solution was also dialyzed and freeze-dried. For brevity, the codes HW, CW, and CS are respectively used to refer to samples extracted with hot water, cold water, and cold saline. Where applicable, numbers representing the ammonium sulfate fraction and letters (I for irradiated and C for control) are used in conjunction with the above code letters.

d) Nitrogen. Each fraction was analyzed for nitrogen by the Kjeldahl procedure. Corrections were made for moisture.

Preparation of protein derivatives from beef.

a) Tryptic digestion. From 0.02 to 1.00 g of Nutritional Biochemicals Corporation trypsin was incubated 2 hr with 5 g of HW-50-C in 0.1*M* phosphate buffer, pH 7.0, at room temperature. The mixture was heated to inactivate the trypsin and dialyzed with water. The dialyzables were pooled and concentrated under reduced pressure. To ensure that no high-molecular-weight material had escaped through the dialysis membrane, the concentrate was dialyzed a second time and the dialyzables pooled and concentrated.

b) Urea degradation. HW-50-C protein samples were incubated overnight at room temperature in 8*M* urea (Szent-Gyorgyi and Borbiro, 1956) buffered with 0.05*M* phosphate buffer, pH 7.0. The reaction mixture was dialyzed with water, discarding the urea, which readily diffused through the membrane before significant passage of the breakdown products. The low-molecular-weight material was also dialyzed a second time.

c) Separation of peptides by fractional electrical transport. The unirradiated peptides, prepared as described above, were placed in the fifth cell of a nine-cell fractional electrical transport apparatus (Roland *et al.*, 1953), the other cells being filled with water. A direct current of 1600 V was applied for up to 48 hours to the system, which was seated in a 4°C water bath. After fractionation the pH of each cell was determined and an aliquot of the contents was irradiated. An aliquot from each cell was also hydrolyzed for 48 hours with 6*N* HCl at 105°C in a sealed tube prior to two-dimensional paper chromatography for detection of amino acids.

d) Amino acetylation. The amino groups of the HW protein preparation were acetylated with acetic anhydride at pH 9.5 (Bello and Vinograd, 1956). The acetylated protein was then adjusted to pH 6.9, dialyzed, and dried from the frozen state.

e) Sulfation. The hydroxyl and sulfhydryl groups of the lyophilized protein were sulfated with concentrated sulfuric acid (Reitz *et al.*, 1946). The sample was then adjusted to pH 6.0 and dried from the frozen state.

f) Performic acid oxidation. The irradiated and unirradiated HW protein samples were treated with formic acid and hydrogen peroxide for 15 minutes at room temperature according to Sanger's method (Sanger, 1949).

Preparation of actin and myosin from beef. The myosin was prepared by extracting fresh minced muscle with 0.5*M* KCl at pH 7.0 and 4°C, followed by dialysis until the protein precipitated. The myosin was reprecipitated to remove impurities. The residue was suspended in acetone to solubilize the actin. Removal of the solvent was followed by extraction of the protein with water and subsequent freeze-dehydration.

Sources of other proteins studied. The collagen was obtained from the Sigma Chemical Co., St. Louis, and the elastin and bovine globin VII from Nutritional Biochemicals Co., Cleveland.

Absolute odor thresholds in aqueous solution. Protein preparations were dissolved in activated-charcoal-filtered water and diluted to give concentrations from 1:10,000 to 1:1,000,000. Odor-panel members were asked to make a paired comparison of each dilution with the water presented in a randomized fashion. The percentages of correct identifications for each concentration were plotted against concentration on probability paper. A best straight line was drawn through the points, and the absolute threshold fixed by intersection of the line with 75% level of correct identifications (Guilford, 1954).

Irradiation procedure. The irradiation was performed in the gamma facility of Argonne National Laboratory, Lemont, Illinois. The samples were sealed in evacuated metal cans (size 300 × 200) and given a dose of 5 megarads over about 200–250 minutes (a rad is defined as the absorption of 100 ergs of radiant energy in one gram of the material being irradiated). The samples were held at 0°F at all times except during exposure in the facility. Irradiation was performed on samples in both the dry and the aqueous state. With one exception, no attempt was made to exclude oxygen.

In this instance the cans were sealed and the gases removed through a punched hole. The cans were then flushed with helium and resealed with solder.

Electrophoretic studies. Electrophoretic patterns of the samples were obtained in pH 8.6 veronal buffer of ionic strength 0.1.

RESULTS AND DISCUSSION

Odor production from irradiated beef protein fractions prepared by ammonium sulfate fractionation. Table 1 summarizes the odors found in ammonium sulfate fractions from beef extracts made in hot and cold water and in a cold saline solution. Each of the samples was found to contain at least 15% nitrogen on a dry-weight basis and was at least 90% homogeneous by electrophoresis studies.

These results indicate that beef proteins vary in their ability to produce an odor when irradiated. This observation confirms that of Bellamy and Lawton (1954), who described various odors produced by a series of known proteins irradiated in the dry state. Even though a precedent has been set for describing odors by such terms as "wet dog" and "wet chicken feather" (Batzer *et al.*, 1959; Bellamy and Lawton, 1954; Burks *et al.*, 1959), as found in Table 1, an attempt was made to relate them to the odors produced when several known proteins were irradiated in the dry state, and subsequently rehydrated. Collagen produced the typical "wet dog" odor exhibited by the HW-25 and HW-50 fractions. This would not be unexpected, because the reflux step used in the preparation would coagulate the proteins and solubilize the collagen by conversion to salt-precipitable glucoproteins (Haurowitz, 1950).

Myosin also produced a "wet dog" type of odor. However, it corresponded more closely to that produced by the CS-50 fraction than to either the collagen or HW fractions. Elastin, with a soapy or bleach odor, bovine globin VII, with a freshly milled cereal odor, and actin, with no perceptible odor, could not be related to any of the odors noted in Table 1. The "wet chicken feather" odor was obtained from the CW-50 fraction, presumably an albumin, because it could be reconstituted in water but not in dilute salt solution. The CS-25 fraction producing the raw irradiated beef odor may have been a globulin because of its salt solubility. The portion of the cold extracts precipitable at above 50% saturated ammonium sulfate concentration, which appears to have contributed little to the odor, would be expected

Table 1. Irradiated [a] odors produced from protein fractions.

Ammonium sulfate fraction	Water extract, 100°C			Water extract, 4°C			10% NaCl extract, 4°C		
	Nitrogen (%)	Yield [b] (g)	Odor [c]	Nitrogen (%)	Yield [b] (g)	Odor [c]	Nitrogen (%)	Yield [b] (g)	Odor [c]
0–25%	16.88	6.8	Some wet dog "A" [d]					1.2	Raw irradiated beef
26–50%	16.77	9.2	Wet dog "A" [d]	16.05	7.5	Wet chicken feather	16.69	13.2	wet dog "B" [d]
51–75%	15.05	1.0	Slight wet dog "A" [d]	15.80	10.9	none	15.74	4.3	none
76–100%	—	0.1	none	15.62	2.8	Sweet		0.5	none
100% soluble	18.27	3.1	Brine	17.58	3.1	Sulfurous	17.30	13.6	none
Weight starting material		28.0			28.3			36.4	
g % recovery		71			86			90	

[a] 5 megarads.
[b] From 1200 g beef.
[c] After addition of water.
[d] Same general odor, but A and B are not identical.

to contain the enzymes and metallo-proteins. This was substantiated by the brown and red colorations of these fractions.

A number of interesting observations have been made concerning the physical and chemical properties of the two principal odors. As previously described (Hedin *et al.*, 1960), the "wet dog" odor is quenched by the addition of a number of reagents that require a hydrogen donor. It has been found in this study that the odor could not be distilled under reduced pressure, and that during the procedure it disappeared from the distillation flask as well. This occurred despite restriction of the temperature of the flask to 20°C, employment of dry ice–acetone traps, and a nitrogen atmosphere. Similarly, after dialysis of the irradiated protein with water, the odor was not found in the dialyzables or the nondialyzables, and could not be extracted from the dried irradiated protein with any of a series of nonaqueous solvents ranging in polarity from methanol to ethyl ether.

With the "wet chicken feather" odor, no such difficulties were encountered. The odor was readily quenched with 2,4-dinitrophenylhydrazine. It was easily extracted with methanol from the dried irradiated protein and was stable when stored in a tight container in the frozen condition.

Absolute odor thresholds in aqueous solution. Absolute thresholds were determined for the "wet dog" and "wet chicken feather" odors for a preliminary assessment of the degree of protein destruction related directly to odor production. Thresholds were found of 3 parts protein in 100,000 parts water for the "wet dog" odor, and 5 in 100,000 for the "wet chicken feather" odor. By comparison with average established threshold levels for offensive compounds such as skatole, pyridine, and ethyl sulfide, it appears that 1/100,000 of the protein was converted to odor-containing compounds.

Tryptic digestion. To learn whether changes in the molecular weight and configuration of the protein determined the nature of the irradiated odor produced, varying amounts of trypsin were incubated under standard conditions with the HW-50-C protein preparation (Table 2). Both the dialyzables and nondialyzables were irradiated,

Table 2. Irradiated odor production as affected by trypsin digestion.[a]

Trypsin concentration[b] (g)	Odor[c]	
	Dialyzables	Nondialyzables
0		wet dog
0.02	wet dog	wet dog
0.05	butyl mercaptan	faint wet dog
0.10	butyl mercaptan-sour	none
0.50	sour-sharp	none
1.00	sour-sharp	none

[a] Two hours at room temperature in pH 7.0 phosphate buffer.
[b] Added to 5 g of protein HW-50.
[c] After irradiation in water with 5 megarads.

and the odor assessed by an informal odor panel.

With the dialyzables, only the samples digested with small amounts of trypsin gave the typical "wet dog" odor. As the enzyme concentration increased, the odor became increasingly sour, comparable to that of an irradiated acid hydrolysate of a protein. With the nondialyzables the odor was noticeable only at the low concentrations of trypsin. At higher levels, no odor of any character was observed. The use of three other enzymes—lysozyme, erepsin, and pancreatin—caused a changed character in the odor of the dialyzables and nondialyzables at low concentration; consequently, they were not studied further.

Urea degradation. The change in the nature of the odor caused by prolonged tryptic digestion was attributed to an extensive degradation of the peptide chain. It has been reported that L-meromyosin could be degraded by 8*M* urea to polypeptides possessing a molecular weight of about 4,600 (Szent-Gyorgyi and Borbiro, 1956). This study was undertaken to determine whether urea would degrade the protein fraction, producing the "wet dog" odor in a similar fashion.

When this was applied as outlined in the experimental section, the irradiated dialyzables produced an odor similar to the "wet dog" odor, as with the low-molecular-weight tryptic digests, but somewhat more sour. The nondialyzables produced a "wet dog" odor but of markedly less intensity than that produced from the trypsin-degraded nondialyzables.

Fractional electrical transport. The dialyzable fractions obtained by treatment of the HW-50 protein fraction with trypsin and urea were separated into 9 fractions with a fractional electrical transport apparatus (see Experimental), and each fraction was subsequently irradiated with 5 megarads. An informal panel was employed to describe the odors produced. The same procedure was applied to an untreated protein to serve as a comparison. Aliquots from each fractional electrical transport cell were hydrolyzed with 6*N* HCl and chromatographed two-dimensionally. The presence of at least ten ninhydrin-reactive compounds in each cell indicated that trypsin and urea treatment of the protein released a large number of heterogeneous peptides. Because fractional electrical transport of the undegraded protein yielded amino acids only in cells 4 and 5, it cannot be inferred that the electric field is responsible for the releasing of peptides.

Table 3 illustrates the considerable variety of odors that can be produced when a series of breakdown products from one source are irradiated. Since the "wet dog" or related odor is found in cells containing slightly acidic material, the previously reported (Hedin *et al.*, 1960) hydrogen donor properties are supported. The other odors are noteworthy in that they compare in acidity or basicity to the isoelectric point of the peptide from which they are formed. These observations provide presumptive evidence for the belief that odor character may be mediated by properties such as size, shape, and amino acid constitution of the peptide chains.

Effect of masking functional groups on odor production. Dried samples of the HW protein preparation were subjected to amino acetylation, sulfation, and performic acid oxidation as described in the experimental section. Two-dimensional chromatography of the hydrolyzed protein derivatives prepared by sulfation and performic acid oxidation showed that the reactions were complete. After irradiation in the dry state, the samples were reconstituted in water and judged for odor character by an informal panel.

In two other experiments, the protein was irradiated as a 33% slurry in 10% saline solution, and in the dry state in a can that had been evacuated and flushed with helium. The results are summarized in Table 4.

These results again demonstrate the variety of odors that can be produced from a single source. They also support previous

Table 3. Odors produced from fractional electrical transport separated protein and peptide mixtures.[a]

Cell No.	HW-50 Undegraded protein [b]		HW-50 Trypsin dialyzables [c]		HW-50 8M urea dialyzables [d]	
	pH	Odor [e]	pH	Odor [e]	pH	Odor [e]
1	2.35	peroxide	1.70	burnt hair	1.57	chlorine
2	2.65	none	1.90	burnt hair	2.08	ester-peroxide
3	3.05	none	2.10	skunk	2.37	methional wet dog+
4	4.28	wet dog++	2.70	methional cabbage	2.48	methional wet dog++
5	5.45	wet dog+	4.10	methional cabbage	4.75	methional wet dog+
6	8.52	none	6.25	methional	11.58	amine
7	9.82	none	9.02	amine	12.05	amine
8	10.60	amine	10.05	amine	12.70	amine
9	11.50	amine- ammonia	11.90	trimethylamine- ammonia	12.75	ammonia

[a] Unirradiated samples were introduced into cell No. 5 and maintained 48 hours in a 1600-V field at 4°C. The nine fractions were individually irradiated with 5 megarads.
[b] 0.5 g of protein.
[c] Obtained from 10 g of protein digested with 0.2 g of trypsin.
[d] Obtained from 10 g of protein degraded with 8*M* urea.
[e] Odor after irradiation.

Table 4. Effect of blocking agents and medium on irradiated odor production of the hot-water-extractable beef protein.

Procedure	Odor [a]
none	wet dog
Amino acetylation	wet dog
Sulfation	slightly musty
Performic acid	sweet ester
10% sodium chloride	fishy
Helium pack	wet dog

[a] After 5 megarads of gamma irradiation.

observations with trapping agents concerning the need for hydrogen donors such as free sulfhydryl groups if the "wet dog" odor is to be produced. The unabated "wet dog" odor obtained with a helium pack suggests that relatively little oxygen is required, if any at all, for the primary odor-producing reaction.

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Uncertainties in Canning Process Calculations

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SUMMARY

The nature of safety factors in canning processes and the uncertainties in calculating lethal values are discussed. The nature, extent, and consequences of these uncertainties are examined. Uncertainties in both the thermal data, obtained from heat penetration measurements, and the bacteriological data contribute significantly to the over-all uncertainty, that in bacteriological data generally being greater. Although experimental error may be large, most of the uncertainty in thermal data is due to real variations from can to can. These uncertainties should be taken into account whenever calculations are made in canning processes, particularly in deciding the safety factor to employ in any particular case.

In many branches of science and technology, in which calculations are made using experimental data obtained with variable material, it is usual to use statistical methods to estimate confidence limits for the figures finally calculated. This is clearly a very useful and desirable procedure when the data and understanding of the nature and causes of variation are sufficient to ensure confidence in the validity of the statistical estimates. In other fields it is more usual to multiply a calculated figure by a safety factor whose choice is often conventional or traditional but nevertheless somewhat arbitrary. The use of more or less arbitrary safety factors seems necessary in many instances for one or both of the following reasons:

1) It is not feasible to apply valid statistical techniques, because the uncertainty in the data cannot be adequately assessed.

2) The safety factor is required for its own sake because the quantity or process being calculated must conform to a standard of safety that cannot be specified very precisely in terms of the quantities calculated.

In canning processes it is probably necessary, in the present state of our knowledge, to think in terms of safety factors rather than precise confidence limits, for both of the above reasons. Safety factors in process calculations are not always explicitly recognized. They are often introduced implicitly by basing the calculations on an assumed initial population of spores with specific properties (e.g., their resistance to inactivation by heat). This hypothetical initial population is probably much harder to destroy than any likely to occur in commercial cans of food. In other cases the standard adopted is the F value, or equivalent time at 250°F, which for any particular pack or can size has been found from long experience to ensure sterility and thus includes an adequate safety factor.

Although it will probably remain necessary to employ somewhat arbitrary safety factors in canning processes, it is desirable to have a knowledge of the uncertainties in calculated lethal values arising from uncertainties in the experimental data. This knowledge would be of value as a guide in choosing appropriate safety factors and varying them to allow for any important differences between packs in the variability from can to can.

This paper is primarily concerned with the uncertainties in the experimental data in process calculations and the effects of these uncertainties on the precision of calculated lethal values. A brief discussion is also given of some sources of systematic errors and some other factors that should be taken into account when choosing safety factors.

CALCULATING LETHAL VALUES

A number of methods exist for calculating the lethal value of a thermal process, but one

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that is particularly convenient for the present discussion is the formula method of Ball and Olson (1957; equation 14.13(a), p. 447). The basic formula of this method is:

$$p = \frac{Z}{F}(fP_h + cP_c) \qquad [1]$$

in which p = percentage lethal heat necessary to sterilize. In the present paper it is merely regarded as a measure of the effectiveness of the thermal process; the reader is referred to Ball and Olson (1957) for a fuller discussion and derivation.

F = number of minutes necessary to sterilize at 250°F,

f = parameter specifying slope of heating curve,

c = parameter specifying slope of cooling curve,

Z = antilog $(T_1 - 220)/z$, in which

T_1 = retort temperature (°F),

z = parameter specifying slope of thermal death time curve,

P_h and P_c are functions of g and z and can be found in tables in Ball and Olson (1957) and Hicks (1958),

g = temperature difference between retort and product at end of heating phase.

It is convenient for present purposes to define quantities occurring in equation 1 in terms of the familiar terminology due largely to Ball and Olson (1957), although it will sometimes be easier to consider errors in D, the decimal reduction time of the organism, than errors in F. It should, however, be stated that some of the definitions could be made more precise and more satisfactory in many respects by recasting them in terms of the theory of the logarithmic order of death as expounded by Stumbo (1948, 1949b), among others.

Equation 1 involves several quantities derived from experimental data, all of which are subject to experimental error. Some of these quantities, such as f, c, and g, are derived from temperature measurements in cans, whereas others, namely, F and z, are derived from bacteriological data. The present paper discusses the experimental errors that commonly occur in estimates of these quantities, and the resulting uncertainties in estimates of p. In addition to such errors caused by the limited accuracy of all measurements there may also be systematic errors associated with the method of calculation and the simplifying assumptions made in deriving the basic equation.

THERMAL QUANTITIES

Considering first the uncertainties arising in quantities derived from temperature measurements, it is known that g and f are related by:

$$g = j\, I\, 10^{-t/f} \qquad [2]$$

in which j = lag factor of heating curve,

I = initial temperature difference between the heating medium and the product,

t = time of heating phase.

Thus j might be regarded as the third fundamental thermal parameter instead of g.

The uncertainties in f, c, and g (or j) arise both from experimental error and from real variations in thermal properties from can to can. In a large number of determinations with cans of beef muscle Hurwicz and Tischer (1956) found an average coefficient of variation of 6.46% in experimental values of the thermal diffusivity, which is inversely proportional to f. Other published data (Jackson and Olson, 1940) sometimes show rather greater variability, with coefficients of variation of 10–15%; still greater variability is found in some particularly heterogeneous packs (Esselen *et al.*, 1951). In measurements made in this laboratory it has been found that the extent of variation in f differs a great deal between different products, but coefficients of variation of 5–10% are common, with values as high as 15% occurring frequently. It does not seem possible, therefore, to use a coefficient of variation in f that would have any general validity, but the use of a coefficient of variation of 7% would not seem unreasonable for the present discussion.

If a coefficient of variation of 7% is estimated from replicate measurements, the 95% fiducial limits of the mean value are [mean $\pm$ $(7tn^{-\frac{1}{2}})\%$] in which t is the appropriate value of Student's t for $(n - 1)$

degrees of freedom. Thus, if $n = 12$, the 95% fiducial limits of the mean are (mean $\pm$ 4.4%).

The uncertainties in f and c arise from two sources: experimental errors and real variations in thermal properties from can to can. The coefficients of variation quoted above include both these types of variation. It would be a cumbersome procedure to compound the experimental error components of f and c in equation 1 in order to estimate the uncertainty in p due to them. However, these generally seem to be small compared with the variation from can to can. This latter source of variation usually affects f and c by approximately the same amount and in the same direction. If it is assumed that can-to-can variation is the major component in the observed coefficients of variation in f, it follows from equation 1, with $f \propto c$, that an uncertainty of 4.4% in f leads to an uncertainty of 4.4% in p.

It is natural to use the mean value of f in studying the relationships between inoculated pack data and calculated p values, as well as for other purposes. The more usual practice when making process calculations, however, is to use the data obtained from the slowest-heating can of a batch (i.e., the can giving the largest value of f).

The greatest value of f observed in a relatively small batch of cans is a quantity whose statistical significance is ill defined. Esselen *et al.* (1951) suggested as an alternative the use of the upper 99% fiducial limit calculated from the mean value of f and its standard deviation.

A calculated standard deviation is a statistic that is itself subject to sampling error, and any estimate of the greatest value of f must be subject to at least as great an uncertainty as the mean; indeed it will almost always be determined less precisely than the mean. Consequently, it would seem best to adopt the procedure, which is usual in most other applications of science, of basing all calculations on the mean values and making use of the calculated standard deviations to estimate the precision of the final result. At present it may not be feasible to do more than take into account the standard deviations in choosing a more or less arbitrary safety factor to be applied to the final result, but even this is to be preferred to a procedure that tends to obscure the considerable uncertainties known to exist.

Values of g (or j) are affected by more of the processing variables than are f or c, and, possibly because of this, there is less evidence on the precision of the estimates of g (or j) than of the other thermal quantities. For simple processing conditions (i.e., zero come-up time, uniform initial temperature in the pack, a homogeneous product, and no variation in retort temperature during processing), the value of j is independent of the thermal diffusivity of the product, for packs heated by conduction, and so should not be affected by most of the can-to-can variations. (A discussion of some of the more important factors that affect j was recently given by Evans (1958), and need not be carried further here.) On the other hand, g is proportional to $10^{-1/f}$ and so *is* affected by variations in thermal diffusivity.

The deviation of processing conditions away from the simple processing conditions mentioned above will, of course, affect both j and g, but such effects can be complicated, making their theoretical estimate an arduous task.

Analyses of experimental data obtained with pumpkin in this laboratory show a coefficient of variation in g of about 2.5% (i.e., the 95% fiducial limits are about equal to the mean $\pm$ 5%). This may not be a very representative figure, but it is interesting to consider its consequences. It follows from tables of P_h (Hicks, 1958) that when $z = 18°F$ and g is about 8°F, an error of 5% in g results in an error of about 8.5% in P_h. The corresponding error in P_c is very similar to that in P_h, so that the resulting error in p is also about 8.5%. It should be noted, however, that the effect on p of a given error in g decreases as the value of g decreases.

Since f and g are determined from the same set of experimental data they are not independent, and the effects on the values of p of errors in these two thermal quantities are not then additive, and exact compounding of the errors would require some statistical investigation.

In addition to experimental error and can-to-can variation there may also be sys-

tematic errors in the thermal data. These include, among others, errors produced by conduction of heat along thermocouple wires (Ecklund, 1956; Cowell *et al.*, 1959), departures of the relevant parts of the heating curves from linearity (Board *et al.*, 1960), and deviations of the cooling curves from the form used by Ball and Olson (1957) in evaluating P_c. The cooling phase is most important and has been discussed, for example, by Hicks (1951) and by Board *et al.* (1960). The latter authors showed that the lethality of the cooling phase for conduction-heated packs was affected by many variables, including type of product, can vacuum, retort pressure, and any delay between steam-off and water-on.

BIOLOGICAL QUANTITIES

If a calculated value of p is to be interpreted as applying to a hypothetical organism for which F = 2.780 min and z = 18.00°F, or some other pair of precisely specified values, there are no experimental errors in F or z to be considered. Though it is often useful to think in terms of such hypothetical organisms, it must always be remembered that practical problems are concerned not with such an ideal case, but with the behavior of real organisms heated in particular foodstuffs.

As in the case of the thermal quantities, uncertainties in the bacteriological quantities arise both from experimental error, due to inevitable imperfections in the techniques used, and from real variations in properties between samples of bacterial spores and samples of foods or culture media. Among the factors causing variations in F (or D) and z are:

(a) the nature of the organism,

(b) the history of the spores, particularly that immediately prior to use; this factor includes the conditions of sporulation,

(c) the substrate in which the spores are heated, and

(d) the substrate used for detecting survivors.

All of these factors can have large effects. It is known that values of D for different organisms at the same temperature vary by many powers of 10, and that resistant organisms that might survive canning processes vary in D values by at least tenfold.

Factors in group (b) are among the most troublesome (Sugiyama, 1951), and their effects can be quite large. For example, Murrell and Scott (1957) showed that controlled drying can vary the D values of spores by factors of up to 10^4, and El-Bisi and Ordal (1956) found that the temperature at which the spores were produced caused variations in D values up to about four- or five-fold. A great deal more study will be necessary before the effects of factors of this sort can be assessed adequately. Indeed, it is not yet certain that all the major factors are recognized.

Our limited knowledge of these factors leaves doubts whether D and z values determined in a laboratory experiment under strictly standardized conditions are the appropriate ones to use in a particular canning problem. It must be expected, at least, that the uncertainties in values applied to a canning problem will be greater than would be calculated by statistical methods from the consistency of laboratory measurements carried out under strictly controlled conditions.

Effects arising from the substrate in which spores are heated are familiar, the effects of pH being perhaps the most spectacular. One of the effects due to substrate that may sometimes be overlooked is that of chemical changes in the substrate during heating. These changes may vary substantially between different processes, and may result in different degrees of spore destruction in heat treatments that process calculations suggest are equivalent.

Effects of the substrate used for detecting survivors have been studied frequently (Amaha, 1952 a, b; Curran and Evans, 1937; Murrell *et al.*, 1950; Olsen and Scott, 1950). The effects of small changes in the composition of substrates can be quite large, and this leads to uncertainties in estimates of the number of spores surviving a given heat treatment. What is measured is always the number of spores that remain capable of growth in a particular substrate, and this may or may not be a good measure of "the number of survivors" in any absolute sense.

Until the reasons for these numerous effects on D and z are understood, it is clearly necessary to be cautious in interpreting laboratory data and not to expect great precision in process calculations.

In process calculations it is usually assumed that z is constant over the relevant temperature range. There is no *a priori* reason why this should be so, but recent work (Pflug and Esselen, 1953; Stumbo *et al.*, 1950) has strengthened the experimental evidence that z is, in fact, usually constant between about 230 and 300°F. Moreover, some significant variations in z that have been observed (Esselen and Pflug, 1956) have been explained by chemical changes in the substrate (i.e., they are probably not really departures from the generally assumed "law" of spore destruction). Nevertheless, the possibility of important variations in the apparent z value with temperature, due to chemical or bacteriological factors, cannot be ignored. Similarly the logarithm of the number of surviving spores need not be exactly a linear function of the time of heating, i.e., the D value at a particular temperature may not be a well-defined constant specifying a uniform property of a sample of spores.

The amount of published data from which estimates of the precision of determinations of z and D can be derived is limited. Pflug and Esselen (1953) carried out a very careful and extensive series of measurements of the rates of destruction of spores of Cameron's putrefactive anaerobic PA3679 heated in neutral phosphate buffer. They found a mean value of z of 16.8°F, with no evidence of variation of z with temperature over the range 235–300°F. Calculations from their data show that the standard error of this estimate of the mean value of z was about 2% of is value. Similar careful measurements by Stumbo *et al.* (1950) showed a slightly, but not significantly, smaller coefficient of variation. Other published data, which are mainly less extensive in some important respects, generally show greater variability. For instance, Kaplan *et al.* (1954) reported a mean value of z of 18.7°F for PA3679, with a standard deviation of 1.67°F (9% of the mean). These data were obtained in experiments with a variety of foods, with culture medium added to the foodstuff for the enumeration of the surviving spores. Variation was much greater when no culture medium was added before incubation.

It is well known that values of D vary substantially with the substrate in which spores are heated, but the evidence for variation in z with low-acid foods is not so clear. Kaplan *et al.* (1954) obtained no evidence that true z values varied with the substrate, i.e., the variation between substrates was not significantly greater than that within replications of the same substrate. On the other hand Stumbo *et al.* (1950), Esselen and Pflug (1956), and others have obtained clear evidence of significant differences between substrates. It seems likely that this apparent discrepancy arises from differences in the precision of individual determinations in the different series of measurements, not to real differences in the extent of variation between substrates.

It is usual, in process calculations, to assume that z = 18°F. Kaplan *et al.* (1954) and many other workers have reported z values that do not differ significantly from 18°F, but Stumbo *et al.* (1950), Pflug and Esselen (1953), Esselen and Pflug (1956), and others have obtained values in various substrates that do differ significantly from 18°F in one direction or the other. It seems reasonable to use a value of 18°F as an approximation in the absence of carefully determined experimental values for any particular case, but this is different from assuming that z = 18°F in any or every particular case. This latter assumption would clearly be quite unjustified.

It is difficult to postulate a representative value of the uncertainty in any estimate of z used in process calculations. The coefficient of variation of about 2% found in some very careful series of measurements no doubt represents substantially greater precision than is usually feasible in data appropriate for a practical case. The coefficient of variation of 9% found by other workers is perhaps more typical, but even greater variability is by no means improbable. It is of interest, therefore, to estimate the uncertainty in p arising from these two coefficients of variation in z.

The terms in equation 1 involving z are Z, P_h, and P_c. The effects of variations in z on P_h and P_c are very similar; in other words, Ball's ρ function (Ball and Olson, 1957; p. 350) varies little with z. Consequently, we may write, as a close approximation,

$$p \propto Z P_h \quad [3]$$

or when the retort temperature is 250°F,

$$p \propto 10^{30/z} P_h \quad [4]$$

Consequently, the table of $10^{30/z}P_h$ given by Hicks (1958) is convenient for estimating the effects of errors in z on p values. When the coefficient of variation in z is 2%, the 95% fiducial limits are, approximately, given by the mean ± 4%. It follows from Hicks' table that when g = 5°F and the mean value of z is 18°F, the 95% fiducial limits of $10^{30/z}P_h$ are 17.31 and 19.15; thus there is, in a sense that will be clear, an average uncertainty of 5% in p. The corresponding limits in $10^{30/z}P_h$ for a coefficient of variation in z of 9% are 13.99 and 22.26 (i.e., an average uncertainty of 23%). It should be noted, however, that the effects of errors in z are less when g is small.

When the retort temperature is 240°F, equation 4 is replaced by:

$$p \propto 10^{20/z} P_h \quad [5]$$

and errors in z seem to affect p more than when the retort temperature is 250°F. This apparent influence of retort temperature is, however, a spurious one, arising from the fact that F is a quantity specified at 250°F that is estimated from the same set of data as z. Consequently, errors in F and Z are not independent and it is only as an approximation that they can be considered separately.

The residual variance in a regression analysis of log D in the data of Pflug and Esselen (1953) was 0.03198. This corresponds to a coefficient of variation in D of 100 antilog $(0.03198)^{\frac{1}{2}}$ or 25%. The coefficient of variation of the mean of n observations would then be $25n^{-1/2}$%. The corresponding coefficient of variation indicated by the sample of the data of Stumbo *et al.* (1950), which was published in full, is $11n^{-1/2}$%. These may not be the best estimates of uncertainty obtainable from the data, but they should be approximations adequate for present purposes.

It is evident that the experimental errors in D are as substantial as those in z, and perhaps even greater. They are also subject to the same doubts about the adequacy of practical conditions as are z values. It follows from equation 1 that p is inversely proportional to F, so that an error in F leads to an equal proportional error in p.

As already pointed out, D and z are normally determined from the same set of experimental data and are not independent. Consequently, the effects of errors in them are not simply additive, and some statistical investigations would be needed to work out exact ways of computing the errors.

GENERAL AND FORMULA METHODS OF PROCESS EVALUATION

As the general method, in its original form, involves graphical integration, the errors of calculation may be significant. The degree of precision obtainable depends on many factors, but it seems feasible to keep the errors of calculation within 1–2% without undue elaboration. The arithmetical variants of the general method described by Ball and Olson (1957) and by Patashnik (1953) employ a somewhat crude numerical integration method (the trapezoidal rule), but there seems no reason to question the conclusion of Patashnik that it is generally feasible to choose a time interval that is convenient and will keep the error of calculation "well within the experimental error or the inherent variations from can to can." More refined numerical integration procedures could be used if the accuracy of the experimental data justified the additional labor. Errors of calculation can, of course, be made very small in applying formula methods.

Errors due to inaccuracy in the bacteriological data affect calculations by the general method in exactly the same way as calculations by formula methods. On the other hand the general method has the advantage that actual heating and cooling curves are used, whereas the formula methods involve simplifying assumptions about the forms of the curves that permit their specification by

a small number of parameters. As pointed out earlier, the theoretical cooling curves are not always good approximations of actual curves (Board *et al.*, 1960; Evans, 1958).

In the author's opinion it is usually preferable to use the general method for products heating by conduction because actual cooling curves are used (Board *et al.*, 1960). With products heating by convection, the greater convenience of formula methods may make them more suitable in most instances. For liquid products the contribution of the cooling phase to the lethal value is small, so that errors in estimating it have only a small proportional effect on the total lethal value.

SOME FUNDAMENTAL POINTS

An important difficulty, affecting formula methods and the general method alike, is that of estimating the number of resistant spores present initially in commercial packs and deciding what level of spore survival can be accepted as a suitable commercial standard.

It is generally recognized that when there is risk of the survival of organisms that may produce toxins lethal to consumers of the product, a very large safety factor, in the ordinary sense, is necessary. It has been estimated that conventional processes based on data for *Clostridium botulinum* implicitly adopt as their standard a reduction in the population of this organism by a factor on the order of 10^{12} or more. When spoilage may occur as the result of the survival of organisms that are more resistant to heat than *Clostridium botulinum* but will not endanger the health of consumers, a much less stringent standard might be adopted; reduction in population by a factor of 10^8 may be adequate in some cases.

Stumbo (1948, 1949a), Gillespy (1951), and Hicks (1951) pointed out the logical necessity for integrating the probability of survival of a spore throughout the volume of the can in order to obtain a true estimate of the lethal value of a process. This integration can be regarded as the calculation of a correction to be applied to the lethal value of a process calculated by the older methods. Hicks (1952) showed that this correction is usually small ($<10\%$ on the p scale) for conduction-heating packs when a conventional *Clostridium botulinum* standard is adopted. It will often be reasonable to neglect the correction in such cases because it can be assumed to be small compared with other uncertainties known to exist. However, this integration correction is larger for products heating by convection, and its neglect would be harder to justify for them. When processes are based on the properties of other organisms and particularly when reduction of the population by a fairly small power of 10 is adopted as the standard, the integration correction may become so large that it would be unwise to neglect it.

CONCLUSIONS

In evaluating canning processes it should be recognized that there are large uncertainties in the values of functions used in the calculations. These uncertainties should be taken into consideration when deciding on the "safety factor" to be employed in each practical case. The thermal quantities used in process calculations are subject to uncertainties believed to arise mainly from real variations from can to can. The uncertainty arising from variability in experimental f values is commonly around 5% on the p scale, and that arising from variations in estimates of g seems generally to be at least as great. Consequently, expression of results in a form that implies precision greater than about 5% is generally unwarranted.

In very homogeneous packs the uncertainty due to variability in thermal properties may be less than 5%, but in more heterogeneous packs it is often very much greater. Consequently, it seems desirable to assess the precision of the experimental data by statistical methods and vary the safety factor applied to the process to take due account of any large differences between products in the extent of variation in properties from can to can. This procedure is to be preferred to that in which calculations are based on the slowest-heating can of a batch studied.

The uncertainties inherent in the bacteriological data used in process calculations seem to have larger effects on the precision of calculated lethal values of processes than

uncertainties in the thermal quantities. A fuller understanding of the causes of variability in the behavior of bacterial spores is, however, necessary before the true degree of precision of the bacteriological data can be estimated with confidence.

Integration of the probability of survival of a spore throughout the volume of the can is equivalent to a fairly small correction factor for conduction-heating packs, adopting a conventional *Clostridium botulinum* standard. It is more important for products heating by convection and has a large effect when processes are based on reduction of the population of a very resistant organism by a fairly small power of 10.

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Compounds Responsible for the Color of Black "Ripe" Olives

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SUMMARY

Isolation and purification is reported of a polyphenol that contributes to the color formation in the processed dark olive, known in commerce as the California ripe olive. This compound (probably oleuropein as described by Cruess) was purified by counter-current distribution. Acid hydrolysis yielded glucose and a second unreported sugar, arabinose. Additional evidence indicates that the compound is not a double ester of glucose, as previously reported.

California canned black "ripe" olives are used extensively as a relish, for seasoning sauces, and for garnishing various foods. Of necessity, these olives are picked green and stored in salt stock for one to several months before being "ripe" processed. Since the olives are intensely bitter, they are put in a lye solution to remove the bitter taste and induce the brown to black color of the "ripe" fruit. Penetration of the lye is stepwise. Between each application the lye is drawn off and the olives are aerated. After the lye has penetrated to the pit it is removed by washing with water. Then the olives are canned in a neutral brine. For further information on this process, see Cruess (1958). One of the main problems is obtaining a consistently dark-colored olive. The processor follows a procedure devised empirically, without clear understanding of why color is good one year and not the next. Until the nature of the pigments is known the processing of dark "ripe" olives will remain an art.

In some of the earliest work done on the chemistry of olives Bourquelot and Vintilesco (1908) obtained an amorphous, yellow powder from all parts of the olive plant. They described this compound, which they named oleuropein, as a non-crystalline, intensely bitter glucoside. In 1934 Cruess and Alsberg purified the bitter principle with a fractional neutral lead acetate precipitation. This amorphous compound was characterized as being free from nitrogen and having a pyrocatechol or orthodihydroxy grouping. On combustion the following C-H values were obtained: C, 53.65; H, 6.51%. Two molecular-weight values were obtained by two different methods: depression of the freezing point in glacial acetic acid, 433; and the boiling point method in alcohol, 470. When Cruess and Alsberg hydrolyzed the glycoside with dilute alkali the bitter taste was removed and a crystalline acid was obtained. The acid showed characteristics similar to that of caffeic acid.

Later Yokoyama and Cruess (1954) isolated the bitter principle by streaking the crude extract horizontally across chromatographic paper and developing the paper vertically (R_f 0.62 in butanol–acetic acid–water 4:1:5). The material was isolated by eluting the separated bands with methanol. This compound was hydrolyzed in dilute mineral acids, and spots appeared at R_f 0.73, 0.83, and 0.18. The first spot (R_f 0.73) gave a strong Molisch reaction, and the R_f 0.18 spot chromatographed with glucose. Alkaline hydrolysis of the compound at R_f 0.62 yielded the same spots, none of which were shown to be caffeic acid.

Shasha and Leibowitz (1959a, b) separated the oleuropein from green olives and recovered two acids from either an alkaline or acid hydrolysis. The first acid was identified as protocatechuic acid (3,4-dihydroxybenzoic acid), and the second as 1-hydroxymethyl-2,6-dimethyl-2-cyclohexenecarboxylic acid, which they named oleuropeic acid. Emulsin was found not to hydrolyze the glucose from the molecule. They concluded

from these data that oleuropein is a diester of glucose.

In 1920 Hilts and Hollingshead, from studies of the chemical changes that occur during the pickling of olives, suggested that the final black pigment was due not to original pigment substances but to something developed during the pickling.

More recently Townsley and Cruess (1954) separated the dark pigments from the "ripe" olives and found more than five dark components, most of which were phenolic substances.

The present study is concerned with a further investigation of oleuropein and its characteristics. Evidence is presented that this compound, which we consider to be a glycoside, plays a major role in the color of dark "ripe" olives.

EXPERIMENTAL

Extraction of sample. Freshly harvested green olives of the Manzanillo variety were pitted by machine and placed immediately in nearly boiling 95% ethyl alcohol. After an initial drop, the temperature was brought back up to 65°C and maintained there for about an hour. The olives were stored in alcohol at room temperature until further use.

Two thousand grams of the pitted olives were macerated in a Waring Blendor in the alcohol in which they had been stored. The slurry was mixed with Hyflo Super-Cell and filtered on a large Buchner funnel. The olive pulp was extracted a second time with 95% ethyl alcohol, and again filtered. The combined filtrate (6 L) was concentrated to about one-third of its original volume with a rotary vacuum distillation apparatus. The concentrated extract (2 L) was put into separatory funnels and placed in a refrigerator overnight. After 12 hours a large amount of oil had formed on the top. This was removed and discarded. The remaining concentrate was then filtered over Hyflo Super-Cell and freeze-dried. This reddish-black, sticky, hygroscopic solid was redissolved in 200 ml of water. The resulting syrup was then extracted in a separatory funnel 9 times with 50 ml of ethyl acetate. The combined ethyl acetate fractions were extracted 5 times with 50 ml of 1*M* KH_2PO_4. The ethyl acetate, which was still reddish-black, was then filtered over a pad of anhydrous Na_2SO_4 and vacuum-concentrated to about 90 ml and stored at −20°C until further use.

Silica-gel column separations. The column technique used was that of Bulen *et al.* (1952) with the following exceptions: Fifteen grams of prepared silica gel was ground with 10 ml of 0.5*N* H_2SO_4 in a mortar. The resulting free-flowing powder was slurried with chloroform and added to a 40 × 1.2-cm column. One ml of the sample from the preliminary ethyl acetate extraction was added to 3 g of silica gel and 1 ml of 0.5*N* H_2SO_4, and, after grinding in a mortar, the mixture was added to the top of the column. Then the following solvents were added sequentially to the column: 65 ml 5%, 150 ml 15%, 200 ml 25%, and 500 ml 35% *n*-butyl alcohol to chloroform. Five-ml fractions were collected and titrated with 0.01*N* NaOH to the phenol-red end point. An identical column was run, and to about every fifth fraction were added 4 drops of KOH-saturated *n*-butanol. The difference in color was measured in a Klett-Summerson colorimeter. The addition of either NaOH or KOH caused these slightly tan fractions to form a dark-brown color.

Magnasol columns. Columns were prepared using 35 g of magnasol (synthetic magnesium silicate; Westvaco Chemical Division, South Charleston, West Virginia) and about 300 ml of acetone. The acetone slurry was added to a column (25 mm I.D.) that, after packing, had an effective length of 100 mm. The sample (about 2 ml in ethyl acetate) was added to the top of the column in a small amount of acetone. The column was developed with the aid of vacuum. Acetone was run over the column until the effluent no longer showed fluorescence as determined with a hand uv lamp. At this point methanol was added, and a brown band moved down. The components of this band were collected and concentrated under vacuum. Paper chromatograms developed with the butanol–acetic acid solvent and sprayed with diazotized sulfonilic acid (Block *et al.*, 1958), and an acetone solution of silver nitrate (Swain, 1953) showed that the acetone-eluted material contained the R_f 0.75 and R_f 0.90 spots, and that the final material eluted with methanol contained only the R_f 0.68 compound as detected with the two sprays.

Counter-current distribution. The counter-current distribution (CCD) apparatus used was manufactured by H. O. Post Scientific Instrument Co., Inc. The robot-operated apparatus is of all-glass construction and has a capacity of 10 ml in each phase.

A partition ratio (k value) of 0.4–0.7 was desired in order to obtain the glycoside in pure form. For this purpose 10 ml of the upper phase and 10 ml of the lower phase were added to two separatory funnels. One acted as the blank, and to the other was added as pure a sample as had been obtained with the other methods of extraction. Since the glycoside has an absorption peak at 280 mμ the amount in each phase was measured with a spectrophotometer. The k value in this case was the optical

density of the upper layer over that of the lower layer. Various solvent mixtures were tried. It was found that *n*-butanol–*n*-heptane–water 1:1:2 gave a k value at 27° C of 0.455. This value was found to vary in a straight-line relationship from 0.439 to 0.465 from 20 to 35°C.

The sample from the magnasol columns was first dried under vacuum and dissolved in 10 ml of *n*-butanol. Five ml was added to the first two tubes, together with 5 ml of *n*-heptane and 10 ml of water. Ten-ml fractions were collected in tubes, and the amount of glycoside in each tube was measured in the spectrophotometer at 280 mμ. The contents of the tubes representing the same peak were concentrated in a rotary vacuum-distillation apparatus. To keep the temperature low and minimize heat destruction, dry ice and ethyl alcohol were used to cool the condensing flask.

The sample was redissolved in acetone and then filtered through an ultrafine sintered-glass filter. The filtrate was evaporated to dryness under vacuum. It was found that if ethanol or water were used as the solvent during the final stages of isolation a large amount of the solvent was retained in the dried product. If a combination of acetone and carbon tetrachloride was used, a dry product resulted. Attempts failed to crystallize the compound from various solvents and combinations of solvents. The noncrystalline powder was slightly tan.

RESULTS AND DISCUSSION

Silica-gel columns had been previously used by the authors to identify the organic acids present in olives (unpublished data). Later in the investigation we became interested in the compound or compounds that cause olives to turn dark in the presence of air and lye. It was felt that one of the compounds separated on the silica-gel column might be a compound contributing to the color. To determine this, two columns were run in an identical manner and their fractions treated as described above.

Fig. 1 shows the results. Three fractions isolated by silica column chromatography turned dark on the addition of alkali. Since it is believed to be the addition of lye and exposure to air that causes processed olives to turn dark, it was felt that these compounds could be responsible for at least part of the color. The second peak seemed to occur in a relatively much greater concentration, so its separation was pursued further.

Silical-gel columns were found unsatisfactory for this separation, for when chromatographed the samples obtained showed, in addition to the glycoside, minor spots indicating the presence of glucose and other breakdown products.

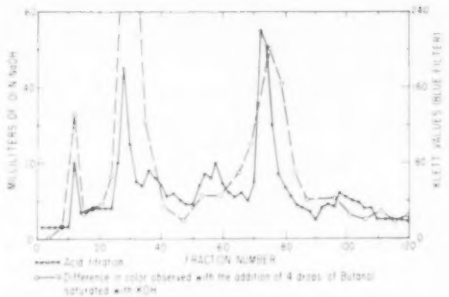
MILLILITERS OF 0.1 N NaOH
KLETT VALUES (BLUE FILTER)
FRACTION NUMBER
Acid titration
Difference in color observed with the addition of 4 drops of Butanol saturated with KOH

Fig. 1. Comparison of the acid titration and reaction to alkali of the fractions from a silica acid column.

Magnasol columns developed with acetone and methanol separated the glycoside from its decomposition products. However, further purification was needed to remove other components eluted in the methanol fractions.

Neutral lead acetate has been used for the isolation of oleuropein (Shasha and Leibowitz, 1959 a, b; Towsley and Cruess, 1954; Yokoyama and Cruess, 1954), but because of the possibility that the glycoside could be altered or broken down in this procedure, other methods were tried. Counter-current distribution has several attributes that recommend it as the method of choice. The

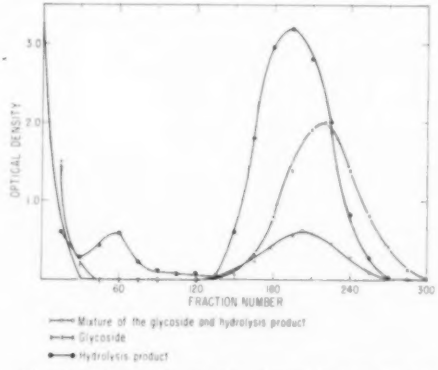

Fig. 2. Separation of the glycoside and its hydrolysis product by counter-current distribution as determined by their absorption at 280 mμ.

solvent need not be subjected to any unusual conditions of temperature, pH, or solvent environment.

Fig. 2 shows the results of the CCD of various samples. The curves show that CCD with *n*-heptane–*n*-butanol–water 1:1:2 does not separate the glycoside (R_f 0.68) and its breakdown produce (R_f 0.75) but that it does separate these from the other components of the mixture. However, the R_f 0.68 and 0.75 compounds could be separated on magnasol columns before the CCD, so these solvents were adequate for purification of the glycoside.

It is possible to calculate the number of the tube that should contain the maximum amount of solute from the following expression (Craig and Craig, 1956):

$$k = \frac{u}{n - u}$$

in which k = distribution coefficient
u = number of tubes in the train
n = number of cycles (fraction number + 100).

The average temperature during the extractions was estimated to be 27°C. Solving for n gave a value of 320, indicating that the maxima should occur in the 220 fraction. This value was obtained experimentally.

The glycoside and its hydrolysis product (R_f 0.75) were hydrolyzed for 48 hours in 3% HCl at 30°C. When these samples were run on paper chromatograms with the butanol–acetic acid solvent a very large spot chromatographing with glucose was obtained from the hydrolysate of the glycoside, and a trace amount from that of the hydrolysis product. Another sugar was also detected in both samples.

Two-dimensional paper chromatograms (phenol–water 80:20 and butanol–acetic acid–H_2O 4:1:5) clearly showed that the second sugar was arabinose. The glycoside was again subjected to the hydrolysis conditions mentioned above, with samples withdrawn at varying time intervals. Glucose was detected in good yield at 0.5 minute, whereas arabinose was detected only after 24 hours.

Molecular weight was determined in a direct and indirect manner. Cyclohexanol was used in the Rast method (Wilson and Heron, 1949) using 42.5 as the molar-depression constant. Azobenzene (m.w. 182.22) was used as the standard, and values of 178 and 183 were obtained. When the method was applied to the glycoside, values of 290 and 282 were observed. Values of 334 and 346 were respectively obtained after drying at a very low pressure for 4 days and 10 days.

The indirect method, based on Dreywood's anthrone reagent (Morris, 1948), gave 32% sugar or an equivalent weight of 562. Two possibilities exist for the true molecular weight. The first is that, since we have two sugars, both could react and the true molecular weight would be over 1,000. The second possibility is that, during the reaction period the arabinose hydrolyzed was insufficient to affect the reading. The results with the time-rate study strongly suggest that the latter is the case. The low results with the cyclohexanol are probably due to water still bound on the molecule.

The infrared spectra of the glycoside and of the R_f 0.75 product were determined. Both samples were run in KBr pellets in a Beckman IR5. The results are summarized in Table 1.

Table 1. A comparison of the IR absorption peaks of the glycoside and its first hydrolysis product.

Glycoside	Hydrolysis product	Probable significance [a]
3378 cm^{-1}	3367 cm^{-1}	–OH group
2915	2924	CH stretching
1706		$\diagdown C = O$
1629, 1575, 1520	1608, 1570, 1520	$\diagup$ Aromatic ring
1441	1443	C-H deformation

[a] Bellamy, 1958.

The infrared data clearly show that when the glycoside is hydrolyzed with dilute HCl the product still retains its aromatic ring and its relatively unchanged C-H stretching absorption. The loss of the $\diagdown C = O$ $\diagup$ absorption on hydrolysis was unexpected and unexplained. The same spectrum is obtained whether the hydrolysis is performed at 100°C for 10 minutes or at room temperature for 3 days.

When the glycoside was treated with a reducing agent specific for aldehydes and ketones such as $NaBH_4$ in water (Anonymous) the $\diagdown C = O$ absorption is lost in the $\diagup$ partially purified product (Fig. 3). These

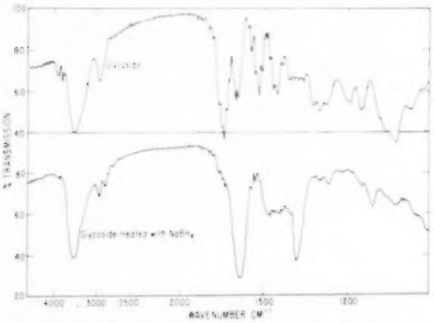

Fig. 3. Infrared absorption spectra of the glycoside before and after treatment with sodium borohydride.

results could also be explained by the formation of an acid salt; however, this would not explain the fact that the $\diagdown C = O$ absorption $\diagup$ is also lost under mild acid hydrolysis conditions. A red color was obtained when the glycoside was treated with concentrated HCl and magnesium, which also indicated that the compound had been reduced.

The glycoside and both of its breakdown products (R_f 0.75 and 0.90) absorb at 283 mμ in 95% ethyl alcohol (Fig. 4). The glycoside has an additional peak at 232 mμ, which is either missing or shifted below 220 mμ in the R_f 0.75 compound. No wavelength shifts were observed for the glycoside when treated with either $AlCl_3$ or anhydrous NaOAc in ethanol. Absorption peaks appeared at 244 mμ and 433 mμ with the addition of sodium ethoxide.

The glycoside turns yellow to red-brown in alkali, and partly loses this color on the addition of acid. In the presence of cold dilute alkali, paper chromatograms show that the glucose is slowly hydrolyzed with the appearance of the R_f 0.75 spot. Two acids can be isolated if the glycoside is boiled in alkali or subjected to alkali fusion conditions. The first acid was identified as protocatechuic acid by co-chromatographing the purified acid with an authentic sample in various solvent systems (R_f 0.75 in butanol–acetic acid solvent). Identical absorption peaks were obtained for protocatechuic acid and the first acid at 259 and 294 mμ. The second (nonaromatic) acid, with an R_f value of 0.87, was not identified and is probably oleuropeic acid. These results are consistent with the findings of Shasha and Leibowitz (1956 a, b).

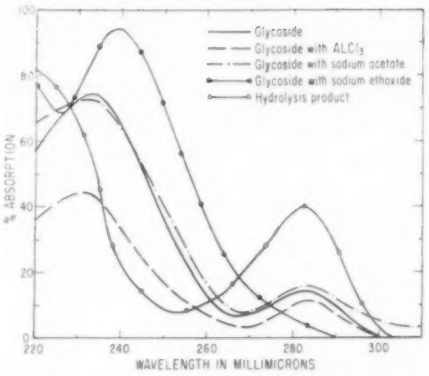

Fig. 4. The uv spectra of the glycoside, the possible wavelength shifts through additions of acids and bases, and the spectrum of the first hydrolysis product.

The carbon-hydrogen ratio was determined on duplicate samples. The ratio for the glycoside was as follows: C, 55.63; H, 6.54; and, by difference, O, 37.88. The hydrolysis product (R_f 0.75) gave: C, 60.86; H, 7.21; and, by difference, O, 31.93.

CONCLUSIONS

These results show that the structure of oleuropein is not the simple double ester of glucose proposed by Shasha and Leibowitz. There is good evidence that another sugar is involved and that this sugar is arabinose. Isolation and purification of the products obtained in an acid hydrolysis proves that these substances are not protocatechuic acid and oleuropeic acid. The R_f values of these two acids are similar to the products of an acid hydrolysis, though both their UV and IR spectra are different. It is also possible to hydrolyze both the R_f 0.75 and 0.90 substances further, obtaining spots correspond-

ing to the two sugars that have been identified.

It is further shown that the two acids are not linked through a double-ester bond, since the $\diagdown C = O \diagup$ absorption band is lost on treatment with $NaBH_4$, with only minor changes in the rest of the IR absorption spectrum. Treatment with acid very easily cleaves the glucose, and the purified residue shows similar properties of solubility, UV spectrum, and IR spectrum except for the loss of the $\diagdown C = O \diagup$ absorption.

These data do not as yet allow a proof of structure, but do provide substance for the characterization of the compound. One of the properties of the compound is the extreme ease with which the glucose sugar may be hydrolyzed. Care had to be taken in the purification procedure not to use heat, even in neutral solutions. Although glycosides are normally not hydrolyzed under basic conditions, we find it not surprising that the compound is partially hydrolyzed under these conditions. Certainly, hydrolysis in base does not prove that the compound is a double ester, in view of the results reported here.

Our results indicate that the aromatic ring and the cyclohexene ring are connected by a carbon chain. The chain is such that alkali fusion oxidizes it to the two acids that have been identified. The position of the $\diagdown C = O \diagup$ absorption shows that the carbonyl is not in conjugation, and therefore not adjacent to the aromatic ring. The apparent loss of the carbonyl absorption after acid hydrolysis could be due to an internal cycloization, possibly through a hemiacetal linkage. Glucose may be in such a position as to offer steric hindrance to such a formation, since carbonyl absorption is lost when it is removed. It is reasonable to suggest that the arabinose is linked to the aromatic ring, because of the difficulty with which it is hydrolyzed.

The formation of the dark color in "ripe" olives is probably due to polyphenol polymerizations. The R_f 0.90 substance obtained on an acid hydrolysis showed the properties of a polymerization product. In the olive the color is probably due to an interaction of several compounds under basic conditions. We have separated three compounds that gave rise to color with base, but there are undoubtedly other compounds in the olive that would give a similar reaction.

Acknowledgments

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Lipolytic Activity of Microorganisms at Low and Intermediate Temperatures. II. Fatty Acids Released As Determined by Gas Chromatography

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SUMMARY

Determination was made of the types and quantities of fatty acids released from lard, tallow, corn oil, and coconut oil by lipases from psychrophilic strains of *Pseudomonas*. The fatty acids were measured by gas chromatography. The rate of fatty acid liberation fell off sharply after the first few hours, but the ratios of the types of fatty acids were not affected until after 24 hours. Additional evidence is presented that much of the palmitic acid in lard is esterified at the beta position. Also discussed is the possible effect of differences in rate of lipolysis of fatty acids from natural substrates on interpretation of their degree of randomness, as measured by enzyme specificity.

Several workers have reported that microbial lipases attack the various natural and pure triglycerides at different rates (Goldman and Rayman, 1952; Mukherjee, 1951; Nashif and Nelson, 1953; Shipe, 1951; Wilcox *et al.*, 1955). Few direct measurements of the fatty acids released have been made, however. Richards and El-Sadek (1949), in an investigation on rancid butter, reported that bacteria produced less volatile acids but more solid acids than molds, and that the liquid acids constituted a majority of the fatty acids recovered. Wilcox *et al.* (1955) employed paper chromatography to measure the individual volatile fatty acids released from butterfat by microorganisms. No quantitative measurements were made.

Recent work by Mattson and co-workers (Mattson and Beck, 1955; Mattson and Lutton, 1958; Mattson *et al.*, 1952) and Savary *et al.* (1957), on pancreatic lipase and triglyceride structure, has indicated that pancreatic lipase is only specific for the fatty acid esterified at the α-position of the triglycerides. Using this position specificity, they presented additional evidence that the fatty acids in natural triglycerides are not randomly distributed.

Improved techniques utilizing gas chromatography (Hornstein *et al.*, 1960) now make possible accurate determination of the individual nonvolatile fatty acids released from natural triglycerides. The present investigation was undertaken to determine how the rate of fatty acid liberation varied with time in bacterial lipolysis, whether the types of fatty acids released from natural fats varied with time, whether the temperature of enzyme production affected its specificity, and whether appreciable differences in strain specificity occurred among selected psychrophilic pseudomonads.

EXPERIMENTAL METHODS

The fat emulsions used were prepared as indicated in the first paper (Alford and Elliott, 1960), except that homogenization was carried out at 4000 lb. with a recycling period of 20 minutes.

Unless otherwise indicated, the bacteria producing the enzymes were grown for 3 days at 20°C in 1% peptone broth buffered at pH 7.0 with 0.05*M* phosphate. After centrifuging at 2500 × G, the supernatant was assayed for lipase activity as previously reported, with the following exceptions. In extraction of the fat and free fatty acids from the samples for titratable acidity and chromatographic

[a] Deceased.

determinations, 10-ml aliquots were taken instead of 10-g aliquots. All extractions were made with two 20-ml portions of petroleum ether. These slight modifications speeded up the procedure considerably without affecting its accuracy. The assay flasks, which contained 1 g of fat (as a 2% emulsion), were incubated 24 hr at 36°C unless otherwise indicated.

The method for separation of the free fatty acids from the fat and their subsequent methylation and determination by gas chromatography is described elsewhere (Hornstein *et al.*, 1960). The quantitative accuracy of this method varies somewhat with both the amount and type of fatty acid ester being measured. For the acids from caprylic through palmitic the method will measure 5–10-μg quantities on the column within ± 5%. For the saturated and unsaturated C_{18} acids, 15–30 μg are required to achieve an accuracy of ± 5%.

The cultures used were strains of *Pseudomonas* from the following sources: **Culture A,** *Pseudomonas fragi*, NRRL B-25, from Dr. W. C. Haynes, NURDD, USDA, Peoria, Illinois; **Culture B,** *Pseudomonas* sp. # 15, from Dr. John Ayres, Iowa State University, Ames, Iowa; **Culture C,** *Pseudomonas* sp. # 35, same source as B; **Culture D,** *Pseudomonas fluorescens*, ATCC 11251, carried in our stock culture collection for several years.

All of these cultures were polar flagellated, nonsporeforming rods, and oxidative in their glucose metabolism, and formed visible growth in peptone broth within 3 days at 1°C.

RESULTS

The previous investigation (Alford and Elliott, 1960) showed that the total amount of lipase produced per cell was greatly reduced when incubation temperature was raised from 20 to 28°C. The data in Fig. 1 suggest that a qualitative effect on the lipase also may occur when the production temperature is increased. There was a small but consistent increase in the percentage of unsaturated fatty acids liberated by the enzyme produced at 28°C, with a slight decrease in the total saturated acids, particularly stearic acid.

As expected, the earlier work showed a quantitative effect of pH on production of lipase. Table 1 indicates that the pH at which the lipase was produced did not affect the specificity of the enzyme. These data also support the evidence in Fig. 1 that elevated temperatures of enzyme production may affect the type of fatty acid liberated.

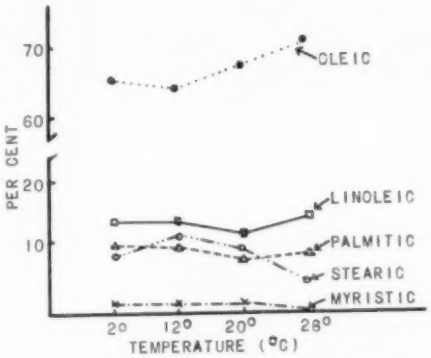

Fig. 1. Effect of temperature of lipase production on its specificity.

Table 1. Effect of pH of production medium on percentage composition of fatty acids released from lard at 2 growth temperatures.

Fatty acid	12°C pH 6.1	12°C pH 6.7	28°C pH 6.1	28°C pH 6.7
Myristic	1	1	tr.	tr.
Palmitic	9	10	9	9
Stearic	11	12	4	5
Oleic	65	64	69	71
Linoleic	14	13	18	15

To determine how rate of acid formation changed during lipolysis, titratable acidity was determined at intervals, using an active culture supernatant as the source of the enzyme. The results are in Table 2. It is apparent that the rate of acid liberation is curvilinear after the first hour or two. After 24 hours, the rate drops about 50–90% below the 1-hr rate. In the case of the lipolysis of corn oil, there was an actual disappearance of acid after 6 days, indicating a loss in some of the acids. The

Table 2. Effect of length of incubation on rate of lipolytic activity of *Pseudomonas fragi*.

	1 hr		5 hr		24 hr		6 days	
	T.A.[a]	Δ/hr[b]	T.A.	Δ/hr	T.A.	Δ/hr	T.A.	Δ/hr
Lard	1.6	1.6	5.8	1.0	10.5	0.3	15.5	0.04
Corn oil	2.0	2.0	6.1	1.0	12.2	0.3	9.2	−0.02
Coconut oil	2.2	2.2	7.6	1.3	12.0	0.2	15.5	0.03

[a] Ml of 0.02*N* NaOH per 10-ml portion.
[b] Change per hour from previous figure.

slightly higher rate of lipolysis of corn and coconut oils than of lard during the first hours of incubation was consistent among different assays, even though the total acid liberated varied.

To determine whether these changes in rate were coupled with changes in the types of fatty acids liberated, aliquots were removed at various intervals and the percentage composition determined. Table 3 shows these data for the fatty acids liberated from lard by *Pseudomonas fragi*. For the first two hours, during which about 5% of the total fatty acids were liberated, the percentage of palmitic acid released was considerably smaller than the percentage in the original fat. Conversely, the unsaturated fatty acids, which constitute only 55% of the fatty acids in the lard, accounted for 79% of the total acids released. The same pattern is apparent after 24 hours. After 6 days, however, when about 60% of the fatty acids had been released, the percentage of palmitic had risen considerably.

Table 4 shows the percentages of fatty acids

Table 3. Effect of length of incubation on the types of fatty acids liberated from lard by *Pseudomonas fragi*.

Fatty acid	Composition of lard mol %	2 hr		24 hr		7 days	
		μg [a]	mol %	μg	mol %	μg	mol %
Lauric	<1	<50	<1	<50	<1	100	<1
Myristic	1	<50	<1	50	<1	1,000	1
Palmitic	29	900	11	1,800	10	20,000	22
Stearic	14	1,200	13	2,100	13	9,600	10
Palmitoleic	2	<50	<1	200	1	2,300	2
Oleic	44	5,900	62	13,200	66	49,300	49
Linoleic	9	1,400	14	2,400	12	15,200	16

[a] Micrograms of fatty acid released per 10-ml portion.

Table 4. Per cent of various fatty acids released from coconut oil, corn oil, and tallow by *Pseudomonas fragi*.

	Coconut oil			Corn oil			Tallow	
	Orig.	2 hr	24 hr	Orig.	2 hr	24 hr	Orig.	2 hr
Caprylic	12	7	9					
Capric	9	11	10					
Lauric	45	37	38	1	1	1		
Myristic	16	28	20	1	1	1	4	1
Palmitic	9	5	11	12	16	17	31	34
Stearic	2	2	2	2	2	2	22	15
Palmitoleic	<1	0	0				1	1
Oleic	5	4	6	28	23	25	39	42
Linoleic	2	5	4	55	57	56	3	8

Table 5. Percentage of different fatty acids released by four strains of *Pseudomonas* [a] from three substrates.

	Lard				Corn oil				Coconut oil			
	A	B	C	D	A	B	C	D	A	B	C	D
Caprylic									<1	5	8	14
Capric									6	9	11	11
Lauric					1				47	35	42	30
Myristic					<1	<1	<1	<1	19	30	16	21
Palmitic	10	9	6	7	12	15	15	14	13	6	9	11
Stearic	14	12	13	13	2	2	1	1	2	2	3	2
Palmitoleic	1	<1	1	1								
Oleic	63	65	63	67	26	25	23	26	7	6	6	7
Linoleic	13	13	14	12	57	58	59	58	6	7	5	4

[a] A, *Ps. fragi*, NRRL B-25; B, *Ps.* sp. #15; C, *Ps.* sp. #35; *Ps. fluorescens*, ATCC 11251.

released from coconut oil, corn oil, and tallow. Again, for some of the acids, there is a variation between the percentages present in the fat and the percentages liberated in the early stages of lipolysis.

To determine whether variations occurred in the types of fatty acids released by different strains of psychrophilic bacteria, four cultures were compared on three substrates. The results are in Table 5.

On lard and corn oil, no real differences were apparent. On coconut oil, some differences did occur. Similar values for these organisms were obtained in other trials, particularly the low caprylic acid value for Culture A and the high value for Culture D. Harper (1957) showed a similar variation in specificity for lipases from different animal sources.

DISCUSSION

The previous work showed that decreasing the temperature at which an enzyme was produced caused a slight drop in the optimum pH for lipase activity. The data presented here indicate that this change in optimum pH was not the result of a change in specificity for any particular fatty acid. Although our primary concern was the effect of low temperatures on enzyme activity, it is interesting to note a slight increase in the amount of oleic and linoleic acids liberated at 28°C, with a corresponding drop in stearic acid. The lack of corresponding effect on palmitic acid may be related to its position in the triglyceride, as discussed later.

As expected in any enzymatic reaction, the rate of fatty acid liberation by bacterial lipase decreases with time. Even so, the decrease appears to be "across the board"; that is, the ratios of the fatty acids liberated remain constant, at least until about one-third of the fatty acids have been liberated.

The differences between percentages of fatty acid liberated and percentages in the original fat may be explained in two ways. There could be specificity of the lipase for the α-position on the triglyceride molecule, irrespective of the fatty acid attached to it. If the fatty acids were not randomly arranged in the fat, a larger percentage of acids esterified at the α-position would appear in the free fatty acids than were present in the whole triglyceride. The other possibility is simply that the lipase might be specific for certain of the fatty acids. Since it is accepted by many workers that fatty acids are not randomly distributed in fats (Mattson and Lutton, 1958; Quimby *et al.*, 1953), the first possibility appears more plausible. Mattson and Lutton (1958) reported a concentration of saturated fatty acids in the β-position in lard, but did not distinguish between stearic and palmitic acids. If one assumes a similarity in mode of action of the lipases from bacteria and from the pancreas, the data presented here give additional evidence of this concentration. The data go further, however, in indicating that a much larger proportion of the palmitic than of the stearic acid is so esterified. Mattson and Beck (1955) reported, after initial hydrolysis of the fatty acids from the α-position by pancreatic lipase, some shifting of the acids from the β- to the α-position. Because of the long incubation period in our studies, such isomerization of the glycerides would be expected. Therefore, continuing lipolysis should increase the amount of palmitic acid liberated. The percentages found at 7 days substantiate this point. The predominance of only palmitic acid at the β-position, rather than of both saturated fatty acids, supports the conclusion of Quimby *et al.* (1953), who, from solvent crystallization procedures, concluded that lard was composed of 2-palmityl glycerides.

Nevertheless, there is still room for doubt that the activity of bacterial lipases (or possibly any lipase) is entirely independent of the structure of the fatty acid. There is considerable evidence on fats and oils that indicates the fatty acids have a limited random distribution. Lack of randomness, however, does not preclude the possibility that the lipase has some degree of specificity, particularly in the early stages of lipolysis. It is quite possible that a combination of positional specificity and fatty acid specificity might combine to accentuate or to obscure the real degree of randomness. The apparent variation in specificity among different strains of bacteria tends to support this, although it would be presumptuous to attach real significance to these strain differences without more detailed evidence of the effect of pH, temperature, etc., on each individual strain.

Another important variable in studies on

natural triglycerides is the triglyceride itself. Relatively wide differences have been reported in percentage composition of any particular fat or oil. It might reasonably be expected that differences in structure among different batches of the same substrate could materially affect the results obtained. Studies are now under way utilizing mixtures of pure triglycerides and randomly rearranged lard to obtain more conclusive data on the effect of chain length, as well as position, on fatty acid specificity.

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Volatile Amines in the Odors of Food

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SUMMARY

To establish whether using steam distillation in the determination of volatile amines in food might lead to artifact formation, a number of nonvolatile nitrogenous substances known to occur in food were tested under these conditions. The results showed that such a formation need be considered only when relatively large amounts of phosphatides, caffeine, theobromine, or hordenine are present in the sample. A method was worked out to eliminate the artificial production of amines from phosphatides. Selected food items were tested for volatile amines following the suggested procedure, and most of the amines were tentatively identified by paper chromatography. In addition to amines, pyrrolidine was probably found in milk and cocoa powder.

During investigations on the odors of food it became necessary to consider the role played by amines in the complex mixtures of volatile compounds. The literature is scant on analysis of the individual volatile amines in food products. Determinations are usually restricted to total volatile bases (Hillig *et al.*, 1958; Pennington and Greenlee, 1910; Thomas and van Hauwaert, 1934; Tomiyama *et al.*, 1956) or to ammonia or a single amine such as trimethylamine, which are investigated individually or separately from the total of other volatile basic compounds (Dyer, 1959; Elkadius and King, 1957; Good and Stern, 1955).

For isolating the basic volatiles from the nonvolatile material, steam distillation under atmospheric pressure and alkaline pH is often applied (Hillig *et al.*, 1958; Stein von Kamiensky, 1957). It has been suggested, however, that under these conditions volatile bases may be produced from proteins or other nonvolatile nitrogenous substances. To eliminate this potential danger volatile bases have also been isolated by room-temperature aeration (Pennington and Greenlee, 1910; Thomas and van Hauwaert, 1934), closed-volume distillation (Conway and Byrne, 1933), or open-volume distillation at reduced pressure and temperature (Schwarz and Thomasow, 1950a; Tomiyama *et al.*, 1956). The latter procedures, however, tend to be time-consuming. Furthermore, bases that may be present within globules of fat may not be as effectively liberated at low temperatures as at higher temperatures, when the fat would be molten and more finely dispersed. Removal of proteins before distillation by precipitation or extraction with trichloroacetic acid (Hughes, 1958), ethanol (Stansby *et al.*, 1944), or $MgSO_4$ (Tomiyama *et al.*, 1956), may also be unsatisfactory, in view of the possibility that volatile base precursors other than proteins may be extracted or bases may be lost by occlusion within undissolved or precipitated material.

Since direct steam distillation at atmospheric pressure has the attractive features of speed of operation and fine dispersion of fats present in the sample, it was considered justified to investigate the reality of the supposed objections against this method. Therefore, a number of single N-containing non-amine compounds known to occur in food, were tested for the formation of amines under the experimental conditions. At the same time a number of food items were investigated by this method to show its practical applicability.

EXPERIMENTAL

Materials. Most of the nitrogen-containing substances (Table 1) were obtained commercially, as were also the compounds mentioned later, in the paragraph on miscellaneous N-compounds. The mixture of phosphatides, however, was prepared from pig hearts according to the method of Klenk and Friedricks (1952) and lecithin was prepared

Table 1. Amines in steam distillates of nitrogen-containing substances.

Compound	Amount in 300 ml buffer	Amines on chromatogram
Proteins:		
Gelatin	18 g	none
Casein	18 g	none
Gliadin	18 g	none
Egg-white of fresh chicken egg	30 g	none
Purine derivatives:		
Caffeine	9 g	methylamine, small amounts ethylamine, trace dimethylamine
Theobromine	4.5 g	methylamine
Adenine	0.3 g	none
Guanine	0.3 g	none
Xanthine	0.3 g	none
Theophylline	0.3 g	none
Phosphatides, etc.:		
Lecithin (from chicken egg yolk)	14 g	none
Phosphatides from pig's heart	± 6.3 g[a]	small amount: methyl-, ethyl-, propyl-, and dimethylamine
Choline	3 g	none
Amino acids:		
19 of the most common amino acids tested separately	3 g	none (in no case any amine found)

[a] Prepared from 300 g of pig heart. The preparation results in two organic solvent solutions that, according to Kler[1] and Friedricks (1952), should contain 6.3 g glycerol phosphatides. The solutions were subjected to the determination as a whole.

from chicken egg-yolk according to the method of Pangborn (1951). For "mixed egg-proteins" the white of very fresh chicken eggs was taken.

The food items tested (Table 2) were all obtained commercially.

Methods. Except for using sodium phosphate buffer instead of Na_2CO_3 to maintain constant pH during distillation (see also Nichols and Foote, 1931), the method followed was that of Stein von Kamiensky (1957).

When single compounds were investigated, a solution or finely dispersed suspension in 300 ml of 0.25*M* phosphate buffer at pH 8.0 was steam-distilled at atmospheric pressure until 250 ml of distillate, trapped in 100 ml of 0.2*N* HCl, was gathered. Food samples, however, often did not reach the desired pH after being mixed with the buffer. In such instances the sample was finely dispersed in 150 ml 0.5*M* buffer, and 1*N* NaOH and water were added until pH was 8.0 and total volume was 300 ml. The distillation procedure was then as followed for single compounds.

In parallel series of tests with food samples, lipides were eliminated before distillation in the following way. The samples were slightly acidified, thoroughly mixed with 100 ml of $CHCl_3$-ethanol (1:1) in a Waring blender and separated by centrifuging. After extracting 3 times, the $CHCl_3$-ethanol extracts containing the lipides and part of the amines were pooled and were shaken with three 50-ml portions of 0.1*N* HCl to recover the amines. The combined HCl extracts, tested and shown to be free of phosphatides, were added back to the extracted food sample. The mixture was brought to pH 8.0 with NaOH and phosphate buffer, again reaching a final buffer concentration of about 0.25*M*. The suspension, now exceeding 300 ml, was then steam-distilled as above.

In all cases Silicone antifoam, tested for negative volatile base production, was used, and conditions were regulated so as to complete the distillation within 30 minutes. Changes in pH during distillation were never in excess of 0.2 unit.

After the distillations were completed the distillates were taken to dryness under reduced pressure (max. temp. 70°C). The residue was taken up in 0.2 ml of water, and 20-μl portions were chromatographed and developed on both untreated and buffered Whatman No. 1 paper according to Stein von Kamiensky (1957). BuOH-AcOH-H_2O (50:1:49) on plain and Na-acetate-treated paper and collidin-H_2O (50:50) on plain paper were the

Table 2. Amines in steam distillates of various foods.

	Amount	Amines on chromatogram
Biscuits (dry wheat)	100 g	small amounts: methyl-, ethyl-, i. butyl-, i. amyl-, and dimethyl-amine
Cocoa powder	100 g	methyl-, ethyl-, dimethylamine; somewhat larger amounts of i. butyl-, and i. amylamine; traces of some tertiary amines. Yellow ninhydrin spot R_f 0.44.
Cocoa waste (tested by direct procedure only)	100 g	traces methyl-, ethyl-, and dimethylamine; somewhat larger amounts of i. butyl-, and i. amylamine; trimethyl-, triethylamine; 1 unknown
Egg (whole)	50 g	Methyl- and dimethylamine; traces of ethyl- and trimethylamine
Haddock (fresh)	100 g	large amounts of trimethylamine; dimethylamine; methylamine (doubtful)
Milk (fresh)	250 ml	methyl-, ethyl-, butyl- and dimethylamine; yellow ninhydrin spot R_f 0.44 (only without $CHCl_3$ extraction)
Pig heart	100 g	traces of ethyl- and dimethylamine
Tinned complete dish of curly kale, potatoes, and smoked sausage	100 g	none

solvents used, and ninhydrin, Na-nitroprusside with acetaldehyde and $NaHCO_3$, Dragendorff reagent, and quinone were applied as sprays. The author's J_2-vapor treatment was not very satisfactory, lacking differentiation between types of amines. It was used only when the presence of tertiary amines was indicated by a negative reaction with ninhydrin and a positive reaction with Dragendorff's reagent. Markers of known amine salts were run alongside the spots under observation on all chromatograms.

To obtain zero blanks it was found necessary to use a very effective steam separator on top of the distillation flask and to eliminate all rubber connections from the glass apparatus; ball-joints were used to reduce the danger of breaking. The apparatus was cleaned by steam distillation at the end of each experiment, and preceding every test a blank distillation and chromatographic analysis was run with all chemicals used in the actual determination; the absence of spots on the chromatograms resulting from these test runs indicated the purity of the apparatus and chemicals with regard to volatile amines.

RESULTS AND DISCUSSION

In preliminary experiments on food the procedure of Stein von Kamiensky was followed closely. In preparing samples for distillation, however, it was found that different amounts of Na_2CO_3 were required for different food items. At the ends of the distillations the pH values of the samples had often risen quite markedly, depending on the amount of Na_2CO_3 added at the onset, so that values were sometimes as high as 10. The danger of artifact formation under these conditions was difficult to assess. No change in pH was observed, however, when the sample had been dissolved or finely dispersed in 0.25*M* phosphate buffer, pH 8, and therefore this modification in the method of Stein von Kamiensky was adopted and used in all the experiments described below.

Table 1 shows the results of the experiments on single compounds. In these tests amounts of compounds were dissolved or dispersed in 300 ml of buffer, corresponding to the maximum amounts reported in the literature for 100 g of any kind of food.

When amine production from any of the compounds tested was indicated by the final chromatogram the experiments were repeated. This time the distillation was continued after the first 250 ml of distillate was obtained, and second and third aliquots of

distillate were gathered, vacuum-dried, and chromatographed. A true amine formation was considered to have taken place when the same or an increasing spot intensity of the consecutive distillate samples was observed on the papers.

It can be seen from the table that volatile amines are produced in very small amounts only from caffeine, theobromine, and mixed phosphatides. Among the latter, lecithin, tested separately, produced no amines.

Ammonia was produced in many instances, proteins being one of the main sources. Ammonium chloride did not interfere with the chromatography of the amines, however, except for very large amounts, which could mask methylamine on the chromatograms. By extraction of the vacuum-dried mixed ammonium and amine salts with hot ethanol, the bulk of the ammonium chloride could be removed by virtue of its insolubility (Schwarz and Thomasow, 1950 b). The ethanolic solution of amine salts could then be concentrated and chromatographed.

In the experiments shown in Table 2, volatile amines were isolated from a variety of food items representing a wide range of specific features. Except with milk and whole egg (respectively 250 ml and 50 g) 100 g of food were investigated.

Since phosphatides had been found to produce small amounts of amines under the test conditions, as shown above, lipides were eliminated from parallel samples of food in duplicate series of experiments by extraction with $CHCl_3$-ethanol.

It can be seen from the table that no amines could be shown to be present in the curly kale dish samples. Aliquots of this food item were therefore used to investigate the effectiveness of the $CHCl_3$-ethanol extraction procedure as follows. Samples were mixed thoroughly with methyl-, dimethyl-, propyl-, and amylamines to make up a concentration of 2 ppm of each of the amines. When the amines were isolated and investigated as described, each appeared on the chromatograms. As judged by the spot areas and intensities in comparison with parallel spots of known amounts on the same paper, no losses in quantity were observed.

It is of interest to mention that, although some of the food samples tested are known to contain phosphatides in appreciable amounts, no difference was observed in the number or amount of amines on the chromatograms, whether or not the lipides had been removed previous to the distillation.

Large amounts of trimethylamine were found in the distillates from fresh haddock. Tarr (1940) showed that trimethylamine can be formed by bacterial enzymes from trimethylamine oxide, which has been found in fish. We do not consider it likely, however, that this amine, found by us in the haddock sample, is formed as an artifact from the same precursor under the test conditions; very strong reducing conditions would be necessary for such a reaction to take place. The conditions under which dimethylamine was formed in the experiments of Hughes (1958) were much more severe than those of our experiments.

Other miscellaneous N-compounds. In addition to the widely distributed N-containing compounds investigated above, some alkaloids and a number of other, mostly less common, N-compounds are known to occur in food. Hordenine is found in the germs of gramineae, pyrrolidine and daucine in *Daucus carota* L., piperine and solanine respectively in *Piper* sp. and potatoes, and betaine in sugar beets. Amounts of 5–9% of piperine were determined in black and white peppers of commerce (Henry, 1939). The maximum concentration of betaine in *Beta vulgaris* is given as 5.1% of the dry weight (Karrer, 1958), whereas the amount of solanine in potatoes may reach 0.01% but is usually 0.002–0.004% (Klein, 1933).[8] Since these compounds are uncommon in food and their amounts are small, there is not much likelihood of misinterpretation of the results arising from this source. Even so, some of them were tested in our experiments.

Piperine and, somewhat to our surprise, betaine did not produce any amine spots on the chromatograms when 300 ml of 1%

[8] When the experimental part of our work was finished the publications of Lepper (1949) and Denes (1956) were brought to our notice, where solanine concentrations in potatoes as high as 0.04% (exceptional) are mentioned.

solutions were investigated, nor did solanine in a concentration of 0.003%. A solution of 1% hordenine, however, showed 3 minute spots of primary amines and a very large tertiary amine spot on the paper.

A major part of the pyrrolidine tested was recovered unchanged from the distillate. The pyrrolidine appears as a single yellow spot on the ninhydrin-sprayed chromatograms on Na-acetate-treated paper with the BuOH-AcOH-H_2O solvent. The R_f value was 0.44 on these chromatograms, and a number of spray tests correlated with a yellow spot found on the same chromatograms of the milk and cocoa-powder experiments, as indicated in Table 2, suggesting that pyrrolidine may be present in these products.

Although the combined information obtained with the different solvents, paper treatments, and sprays lends confidence to the identification of the compounds, the identifications in Tables 1 and 2 should be considered tentative.

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Studies on Some Aspects of Custard Apple Peroxidase

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(Manuscript received July 26, 1960)

SUMMARY

A purified peroxidase enzyme solution was prepared from custard apple pulp, and its characteristics studied. Its catalytic effect on oxidizing various aromatic amines and phenols was reported. Ascorbic acid oxidase is shown to be absent in the enzyme extract. The influence of temperature, pH, and concentrations of sulphur dioxide, ascorbic acid, sucrose, potassium cyanide, and hydrogen peroxide on the enzyme activity are indicated. The "PZ" value for custard apple pulp was found to be 0.06 on dry basis.

During studies (Bhatia *et al.*) on preservation of the pulp of custard apple *(Anona squamosa)*, it was observed that the pulp turns pink on exposure to air. The numerous references to such behavior in the literature mostly ascribe the change to the presence of oxidases. Hussein and Cruess (1940) reported peroxidase as being responsible for the brown color formed when freshly expressed, unheated juice from Vinifera grapes was allowed to stand in air. Jimenez (1947) studied in detail the characteristics of peroxidase, shown to cause browning in cut surface of guava. Cruess *et al.* (1932) indicated the presence of peroxidase in peach tissue and demonstrated its effects on browning in canned peaches. Since peroxidases from different sources vary widely in behavior toward individual substrates and in other properties, work was undertaken to study the characteristics of the purified enzyme preparation obtained from custard apple pulp, for which such information is not available.

EXPERIMENTAL

Preparation of enzyme extract. Custard apple pulp stored at 5–10°F in plain cans was used. The enzyme extract was prepared essentially by the method used by Jimenez (1947). The thawed pulp (800 g) was filtered through cloth, the residue washed with 100 ml ice-cold water, and again filtered. Both the filtrates were combined and the enzymes precipitated by adding two volumes of acetone at 5°F. After allowing to stand for 10 min, the enzyme precipitate was filtered through coarse filter paper (No. 41). The precipitate was macerated with 200 ml of ice-cold water and then filtered. To the filtrate containing the enzyme were added two volumes of acetone at 5°F. After an additional 10 min the precipitate was filtered and the precipitated enzyme suspended in 150 ml of ice-cold water. This operation was repeated thrice, and the purified enzyme solution thus obtained, a white suspension in water, was kept at 5°F and used for the following studies after suitable dilution.

Methods of estimation. McIlvaine's citrate-phosphate buffer (pH range 2–8) was used. Freshly distilled guaiacol was employed as substrate, and the procedure suggested by Hussein and Cruess (1940) was adopted with a few modifications. To 17 ml of the buffer was added 1 ml of suitably diluted enzyme solution followed by 1 ml of 1% guaiacol solution in alcohol and 0.5 ml of hydrogen peroxide (0.1*N*). After gentle mixing and leaving the contents 20 minutes at 30°C, 10 ml of dilute acetic acid (50% v/v) was added to arrest the reaction, and the developed color was measured with a lumetron colorimeter, employing 53 filter. In all cases suitable dilutions with acetic acid (50% v/v) were made so as to get the optical density values in the accurate range of the instrument (O.D. less than 0.6). However, all reported values were calculated taking the dilution factor into consideration. Since it was observed that the brown fades if exposed to light in acid medium, the reaction was carried on in amber-colored bottles to minimize such losses, and the color readings were taken immediately. Even in amber-colored bottles, color losses of 10–20% were noticed during ½ hr after stopping the reaction. It was also noted that addition of an equal volume of absolute alcohol to the reaction mixture inhibits the enzymic activity and that the color under these conditions is more stable than in acetic acid medium.

Determination of PZ value. It has been generally considered that a plant enzyme that catalyzes the oxidation of pyrogallol to purpurogallin is a peroxidase. Peroxidase activity has been customarily expressed in terms of mg purpurogallin formed per mg of pure enzyme or per mg of material containing the enzyme and referred to as PZ number, under specified conditions. The details of the method as adopted in the present investigation are those reported by Sumner and Gjessing (1943). Two ml of fresh 4% pyrogallol, 2 ml of 0.5*M* phosphate buffer of pH 6.0, 15 ml of water (or less, if a large volume of enzyme is used), and 1 ml of 1% H_2O_2 are mixed in a 125-ml Erlenmeyer flask, which is placed in a thermostatic bath at 20°C. When the contents attain the temperature of the bath, 1 ml of properly diluted purified peroxidase solution is added and the contents mixed. After 5 minutes, 1 ml of 2*N* H_2SO_4 is added to suppress the reaction, and the purpurogallin is extracted with 3 or 4 portions of ether. The ether extracts are combined, filtered through a dry filter, and made up to a known volume.

The intensity of the yellow color of the ether extract is measured in the accurate range with a lumetron colorimeter using filter 42. For reference standard, purpurogallin was prepared by reacting custard apple pulp with pyrogallol and hydrogen peroxide, and the absorption of known strengths of purpurogallin was determined. The PZ value of the stored custard apple pulp was thus calculated.

RESULTS AND DISCUSSION

Action of various substrates. The action of the purified enzyme preparation on various substrates was tested, using a buffer of pH 5.5. After addition of hydrogen peroxide, the enzyme gave a blue color with benzidine, brown with catechol, buff-colored precipitate with α-naphthol, purple with β-naphthol, faint lemon-yellow with *o*-cresol, brownish-yellow with gallic acid, brownish-red with hydroquinone, brown with guaiacol, yellow with pyrogallol, red with orcinol, and intense purple changing to blood-red with *O*-phenylene-diamine. Phenol gave a faint reddish-brown on long standing, whereas addition of tyrosine did not produce any precipitate or coloration, indicating absence of tyrosinase. The enzyme did not oxidize resorcinol, and differed from horseradish peroxidase in this respect; p-cresol, m-cresol, tryptophan, and phloroglucinol were not affected by the enzyme preparation.

Dehydrogenase tests. In the presence of ascorbic acid in a Thunberg tube under vacuum, the purified enzyme solution caused no noticeable decolorization of methylene blue in 6 hr, indicating the absence of ascorbic dehydrogenase.

Optimum pH. The intensities of color produced by the enzyme with guaiacol and hydrogen peroxide were determined at pH 3.0, 3.4, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 (Table 1). The enzyme activity was highest around pH 5.0, and was reduced considerably around pH 3.0.

Table 1. Effect of pH on enzyme activity.

pH	Optical density (53 filter)
3.0	0.005
3.4	0.007
4.0	0.240
4.5	0.560
5.0	0.780
5.5	0.760
6.0	0.680
6.5	0.580
7.0	0.470
7.5	0.340
8.0	0.200

Effect of SO_2 concentration. Though the optimum pH for the enzymic activity was rated to be highest at pH 5.0–5.5, the effect of various concentrations of sulphur dioxide added in the form of potassium metabisulphite (KMS) was determined at pH 4.0 to correspond to the pH of the pulp (Bhatia *et al.*, 1961) as standardized to preserve custard apple pulp. Guaiacol and hydrogen peroxide were added 10 min after allowing the enzyme solution to be in contact with the KMS solution. A gradual decrease in the intensities of the color was noted with increasing concentrations of sulphur dioxide, and a sulphur dioxide concentration of 350 ppm failed to produce any color and inhibited enzymic activity completely.

Ascorbic acid concentration. Addition of ascorbic acid at levels ranging from 1 mg per cent to 50 mg per cent to the reaction mixture consisting of enzyme, guaiacol, and hydrogen peroxide in buffer of pH 4.0 showed complete inhibition of color even after 24

hours in samples containing ascorbic acid in excess of 25 mg per cent. The color developed immediately when ascorbic acid concentration was kept at 1 mg per cent, whereas at 12 mg per cent it took about 10 min for the color to appear.

Sucrose concentration. Buffer solutions (pH 4.0) containing sucrose equivalent to a final concentration of 20, 30, 40, 50, 60, and 70% solution in the reaction mixture (containing enzyme + guaiacol + H_2O_2) were tested for their effect on the enzymic activity as judged by the intensity of color produced. The results (Table 2) show that

Table 2. Effect of sucrose concentration on enzyme activity.

Sucrose concentration (° Brix)	Optical density (53 filter)
20	0.78
30	0.72
40	0.63
50	0.54
60	0.36
70	0.15

the increased sucrose concentration retards enzymic activity.

Effect of variations in enzyme and substrate concentrations. There was a good correlation between the color production and increase in enzyme concentration up to a particular concentration (Fig. 1). A fair linearity up to 2.0 ml of enzyme in 20 ml reaction mixture was observed, while with increasing enzyme concentration in spite of sufficient substrate, the proportionality between enzyme concentration and color development did not hold good.

The effect of the concentration of substrate (guaiacol) in the reaction mixture on the amount of color formed was linear up to a certain concentration, as illustrated in Fig. 1. The reaction mixture contained 0.1, 0.3, 0.5, 0.7, 1.0, 2.0, and 3.0 ml of 1% guaiacol solution, together with the enzyme and hydrogen peroxide solutions in a total volume of 20 ml at pH 4.0. The color development was proportional to the quantity of substrate up to a concentration of 0.7 ml, with a slow increase thereafter under the conditions of the experiment.

Hydrogen peroxide concentration. Since the concentration of hydrogen peroxide has been known to influence the enzymic reaction, the activity of the enzyme solution was determined with varying concentrations of hydrogen peroxide. Considering as 100 the activity with a concentration of 5×10^{-3} g mols per liter of H_2O_2, the activities under the conditions of the experiment were shown to be 50 at 1×10^{-3} g mols per liter; 42 at 1×10^{-2} g mols, 12 at 5×10^{-2} g mols, and very faint at 7.5×10^{-2} g mols.

Optimum reaction temperatures. Buffer solutions of pH 4.0 (17 ml) were adjusted to the following temperatures: 20, 25, 30, 35, 40, 50, and 60° C. The enzyme suspension, guaiacol, and hydrogen peroxide were then added, and after maintaining the reaction mixture for 20 min at each of the temperatures, the reaction was inhibited by addition of acetic acid and the color intensities compared. The results (Table 3) show that 20–30° C appears to be optimum temperature for this enzyme, as against 36.3° C for grape peroxidase (Hussein and Cruess, 1940).

Heat resistance. The purified enzyme preparation was separately kept for 5 min at 30, 40, 50, 60, 70, 80, and 90° C, and the activity was tested with guaiacol and hydrogen peroxide. The activity did not decrease up to 70° C, and was lowered thereafter. When kept 30 min at 70° C, the activity was reduced to nearly 50%, while it was reduced

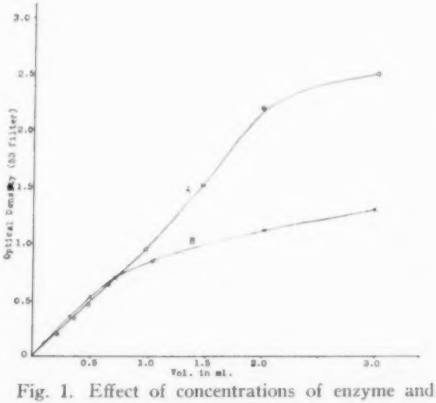

Fig. 1. Effect of concentrations of enzyme and substrate on color development. A) Volume of enzyme (ml); B) Volume of substrate (ml).

Table 3. Effect of reaction temperature on enzyme activity.

Reaction temp (°C)	Optical density (53 filter)
20	0.900
25	0.900
30	0.875
35	0.680
40	0.540
50	0.155
60	0.005

to about 90% when kept 30 min at 80° C. Inhibition was complete at 80° C for 60 min, or at 90° C for 30 min, which was regenerated slightly after 5 hours and up to about 20% after 24 hours. Horse-radish peroxidase is known to behave likewise.

Inhibition by potassium cyanide. The inhibitory effect of different concentrations of potassium cyanide on the enzymic activity has been studied. Considering the activity as 100 without added potassium cyanide, the respective activities were 67, 43, 16, and 2 for KCN concentrations of 5×10^{-7}, 2.5×10^{-6}, 5×10^{-6}, 1×10^{-5}, and a concentration of 2.5×10^{-5} suppressed the activity completely.

PZ value. Stored custard apple pulp was adequately diluted, and, after filtration through a coarse filter paper, the purpurogallin formed with pyrogallol and H_2O_2 was extracted with ether. The color of the ether extract was compared with a solution of known concentration of purpurogallin in ether. From these values, it was calculated that the stored pulp has a PZ value of 0.012 (0.06 on dry basis), as against a PZ value of 0.8 for horse-radish when dry, reported by Willstatter (Sumner and Somers, 1947).

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Sugar Sulfonates and Their Behavior

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SUMMARY

New crystalline bisulfite addition products of mannose and galactose were prepared, namely α-hydroxy-galactose sulfonate and α-hydroxy-mannose sulfonate, and their properties studied. In addition, the rates of dissociation of these sulfonates were studied, and further evidence is presented to show that these products have an open-chain configuration.

The evidence obtained supports the theory put forward previously by one of the authors, namely, that sulfur dioxide and sulfates interact only with the open-chain aldehyde configuration of the sugars when the cyclic forms of the sugars open during their mutarotation. A maltose sulfonate was prepared in which only half of the molecule was capable of interaction with sulfites. This addition product failed to crystallize. It is probable that the same mechanism is of importance in interpreting the browning phenomena during the Maillard reaction.

The chemistry of sulfur dioxide, sulfites and their addition products with various sugars, namely the α-hydroxysulfonic acids, was summarized by Joslyn and Braverman (1954). The formation of such addition products plays an important role in the preservation of fruit and vegetable products with SO_2 or sulfites.

It is well known that many sulfited food products have their SO_2 in two forms: the so-called free SO_2, which is estimated by direct titration with iodine, and the bound SO_2, which can be determined only after saponification and subsequent acidification. Kerp (1907) was the first to suggest that SO_2 or sulfites combine with glucose and arabinose to give rather unstable addition products; he was able to prepare the first of the series of α-hydroxysulfonic acids, glucose sulfonate, in a rather crude form, and studied its dissociation constants. Other workers showed that various sugars exhibit different degrees of affinity for SO_2, but it has been shown that sucrose and fructose are incapable of forming such addition products, the first because it lacks a free carbonyl group, and the second because of its peculiar state of mutarotation (Braverman, 1953).

Those engaged in processing fruit-juice concentrates or other food products preserved with sulfur dioxide are aware that only the amount of SO_2 that remains free is capable of exerting its bactericidal action, and that the more concentrated the product, the more of the ineffective bound SO_2 is formed during storage.

A somewhat similar situation exists when browning phenomena are caused by the interaction of sugars with amino acids (the Maillard reaction). There, in the first stages, the amino group plays much the same role as the bisulfite during formation of the sulfite-addition product. Such sugar–amino acid systems have been studied by Frankel and Katchalsky (1937–1941) and their findings presented in a series of papers. They also found no evidence of any reaction between amino acids and nonreducing sugars or ketoses (sucrose, fructose, or raffinose), whereas in the case of aldoses some combination took place. The optimum combination occurred at pH between 7 and 9; at high temperatures the reactions with sugars are different from the simple amino-aldehyde condensation.

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Both the formation of sugar-hydroxysulfonic acids and the sugar–amino acid condensation proceed at first very slowly, and only as time goes on, as well as in very concentrated solutions, do these reactions take place at an accelerated pace.

This strange behavior of the aldoses in both cases led Braverman (1949) to suggest that SO_2 is capable of combining only with the open-chain aldehydic form of a hexose, such as is present only to a very slight extent in an aqueous solution in equilibrium with α- and β-D-glucose during the mutarotation of glucose. The figures found for the open-chain aldehydic forms in solutions of various sugars, at pH 7 and 25°C, are as follows (in mole-per cent) (Pigman and Goepp, 1948): glucose, 0.024%; mannose, 0.064%; galactose, 0.082%.

Once the equilibrium between the α and β configurations and the open-chain aldehydic form of the sugar has been disturbed by the combination of the latter with a sulfite, more of the cyclic forms will open, giving more of the true aldehydic form, which proceeds to bind additional SO_2.

This theory was examined by Braverman (1953). In his study a method of preparing glucose and arabinose sulfonates of high purity was described in detail. This consists of mixing slowly, with constant stirring at 40°C, equimolecular weights of the sugar and sodium bisulfite dissolved separately in minimum quantities of water. The resulting solution, at first cloudy, is maintained at 30–35° in an incubator with a slowly moving stirrer. It becomes clear after an hour or so, and after 50–60 hr becomes turbid with suspended crystals of sodium glucose sulfonate. After 3–5 days, an abundant crop of crystals is obtained, filtered on a Büchner funnel, and washed first with small portions of 75% methanol, then with 99% methanol and ether, and finally dried under vacuum.

Sodium glucose sulfonate prepared in this way crystallizes (Fig. 1) with one molecule of water, melts at 92–93°C, $[\alpha]_D^{25} = -6°$, is insoluble in nonpolar solvents and very soluble in water, with partial dissociation into glucose and sodium sulfite.

Braverman attempted to explain the mechanism of the interaction between sulfur dioxide and sugars containing a free carbonyl group: Good evidence has been presented for the structure of glucose and arabinose sulfonates as open-chain compounds, based on experimental results from elementary analyses coupled with hydration of the addition products, measurement of the infrared absorption spectra indicating the absence of a C–O–C bond, and determination of optical rotation indices.

Fig. 1. Sodium glucose sulfonate crystals (×44).

Attempts failed to combine fructose with SO_2 at ordinary temperatures, thus supporting previous findings. It is considered that this phenomenon is due to mutarotation of fructose proceeding along quite different lines from that of glucose.

The present work was conducted to prepare, by similar reactions, the α-hydroxysulfonates of galactose, mannose, and maltose, determine their dissociation constants, and confirm the suggested mechanism.

EXPERIMENTAL PROCEDURE

1) The addition compounds were synthesized by the technique described above, using $NaHSO_3$ for glucose (and with larger amounts of water) for galactose, mannose, and maltose.

2) The free-bound SO_2 ratio was determined in order to follow the degree of interaction by successive titration of small samples against 0.01*N* iodine in the presence of starch, with the solution first acidified with normal HCl (free SO_2) and then rendered alkaline with an excess of 2*N* NaOH and reacidified for the second titration (bound SO_2).

3) The *optical rotation* was determined polarimetrically (at 25°C, using 20-cm cells). That of the hydro-sulfonic acids was measured in acidified

solutions (3*N* and 0.6*N* HCl) to prevent quick dissociation.

4) The molecular weight was determined on samples dried overnight at room temperature: a) by iodometric titration (using 0.1*N* iodine), and b) from the specific optical rotation of the decomposed compound.

5) The *water of crystallization* was determined: a) with the Karl Fischer reagent (used in excess and titrated back with standardized methanol-water mixture), and b) by overnight drying in vacuo, first at room temperature and subsequently at 80°C.

RESULTS AND DISCUSSION

Of the three sugars tested in the present study, galactose and mannose gave corresponding α-hydroxy-sulfonic acids, well crystallized and of high purity. Sodium galactose sulfonate crystals, after being purified with methanol and ether (Fig. 2), melted at 145°C, with decomposition. Similar crystals were obtained when preparing sodium mannose sulfonate (Fig. 3).

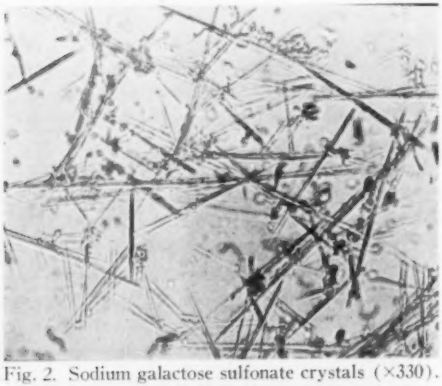


Fig. 2. Sodium galactose sulfonate crystals (×330).

Fig. 3. Sodium mannose sulfonate crystals (×44).

In both cases the preparation of pure crystals is rather more difficult than in the case of the glucose compound. This is due mainly to their relatively higher binding power to sulfur dioxide: the formation of the addition products is much more rapid in this case than in the case of glucose, and at the appropriate concentration the crystals are apt to create a compact mass occluding the mother liquor as well. It was necessary to use 70% methanol first in order to separate the crystals from the mother liquor, followed by washing with absolute methanol and ether.

The degree of affinity of the sugars studied is compared with that of glucose by the following series:

mannose > galactose > glucose
> arabinose

This series differs slightly from the series proposed by Berg or by Ingram and Vas (cf. Braverman, 1953); however, it should be noted that in each case different concentrations were used during the interaction of SO_2 with the sugars.

In the case of maltose no crystals could be obtained. The degree of combination in this case was measured as per cent of glucose groups in the maltose bound to SO_2 (see Fig. 4).

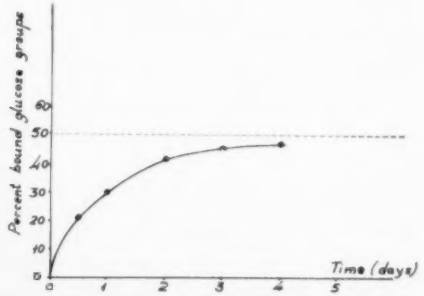
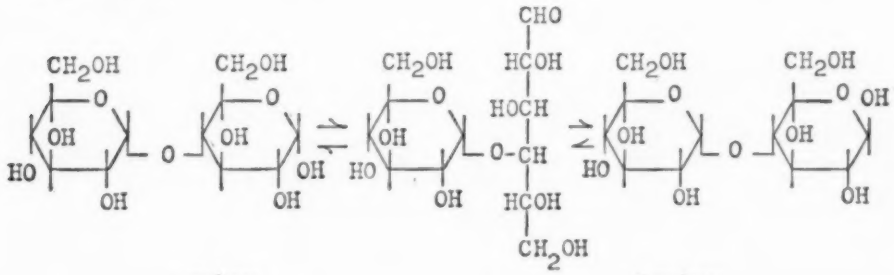


Fig. 4. Degree of association of maltose with SO_2.

The bisulfite was added in the proportion of 2 mols of $NaHSO_3$ to 1 mol of maltose, though it should be clear that only one glucose group of the maltose molecule, that with the free carbonyl group, is capable of combining with SO_2 whereas the other is bound in a glucosidic bond of the maltose to another molecule of glucose. At the end of

each experiment the per cent of glucose groups that entered the reaction was determined by the usual method of titration with iodine for free and bound SO_2. A maximum level of combination (about 48% of glucose groups that entered the reaction) was arrived at in 5 days, but this maximum could not be exceeded; on complete evaporation a solid transparent mass was formed without any trace of crystals. Consider the structural configuration of maltose during its mutarotation in solution:



α-maltose β-maltose

One may assume that the open chain of only half of the disaccharide molecule of maltose undergoing mutarotation is alone capable of taking part in the reaction.

Specific optical rotation. Using better instruments, the $[\alpha]_D^{25}$ for sodium glucose sulfonate should be corrected to −7.0, instead of −6.0, as found previously (Braverman, 1953).

The specific optical rotation for all addition products was low compared with the corresponding sugars (as shown in Table 1), providing further confirmation of the open-chain reaction theory, since most open-chain sugar derivatives generally have a lower specific optical rotation than the corresponding sugars.

Dissociation of addition products. On the basis of the polarimetric measurements, dissociation graphs were plotted for the glucose and galactose products (Fig. 5). A linear relationship was found between reaction time and the logarithm of the concentrations of the undercomposed addition product, indicating that the reaction of dissociation is one of first order.

For a reaction of first order one may write:

$$-\frac{dC}{dt} = kC \text{ or } \ln C = -kt$$

in which C = concentration, t = time, k = velocity constant.

The velocity constants of the dissociation of glucose and galactose hydroxysulfonates, and their half-life, were calculated from the slopes of the time-log concentration curves. These are given in Table 2. They show that the galactose-sulfite compound is as much as 3–3½ times as stable as the corresponding glucose compound.

Table 1. Specific optical rotation of the sugars and their addition products.

Sugar	S.O.R. of sugar	S.O.R. of addition product
Glucose	+52°.7	− 7°.0
Galactose	+80°.2	+10°.07
Mannose	+14°.2	± 0.0

Table 2. Dissociation of glucose and galactose sulfonates.

Addition product	k	t/2 (hr)
Na-glucose sulfonate in 3*N* HCl	0.0720	9.8
Na-glucose sulfonate in 0.6*N* HCl	0.0218	29.3
Na-galactose sulfonate in 3*N* HCl	0.2500	2.8
Na-galactose sulfonate in 0.6*N* HCl	0.0680	10.2

Molecular weight and water of crystallization. Both the Karl Fischer and vacuum drying methods gave the percentage of hydration of the obtained addition products as

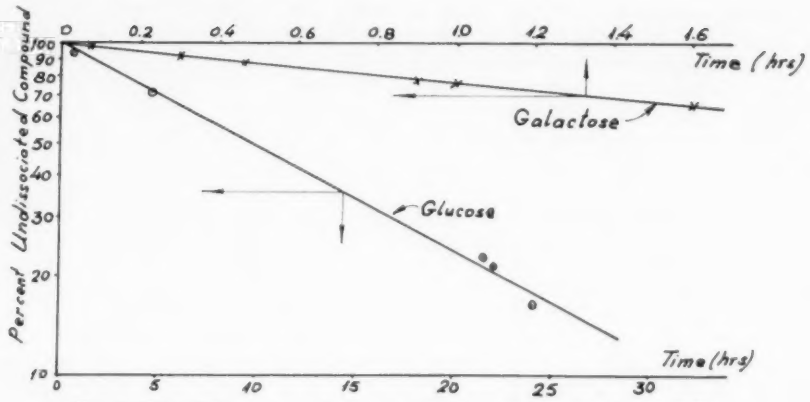
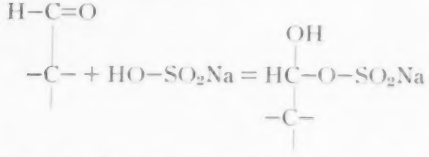


Fig. 5. Dissociation curves of glucose and galactose sulfonates in 3*N* HCl.

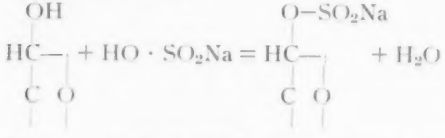
10.7 to 11.7%. This value corresponds to two molecules of water of crystallization.

The average molecular weight of galactose sulfonate obtained by the two methods, iodometric titration and the specific optical rotation, was 320, which gives the value of 284 for the dehydrated product.

These results confirm again the findings of Braverman (1953) that the anhydrous addition product of glucose with SO_2 has a molecular weight of 284, when considered as an open-chain compound:



and only 266 if the product would be the result of the interaction with the cyclic form of glucose, as follows:



Thus, anhydrous galactose-sulfonate is found to have a similar molecular weight, namely 284, with two molecules of water of crystallization.

In conclusion it may be stated that the results in respect to specific optical rotation and molecular weight confirm the open-chain structure suggested for the sugar-SO_2 addition compounds.

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Studies on Frozen Fish. I. Denaturation of Proteins

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(Manuscript received August 1, 1960)

SUMMARY

The effect of frozen storage and different glazes on the denaturation of fish proteins was studied. The solubility of proteins was affected less when glazes were used. The denaturation of proteins was restricted to the actomyosin fraction of proteins; the sarcoplasmic fraction remained unchanged. The total actomyosin denaturation was not closely related with either the decrease in apyrase activity of the muscle brei or with the contractility of the muscle fiber.

Loss of tenderness and the characteristic fish flavor are the important changes associated with the freezing and cold storage of fish (Dyer, 1951). Loss of the characteristic fresh-fish texture has been attributed to denaturation of fish muscle proteins. Reay (1933) found that the solubility of muscle proteins in salt solutions decreased after frozen storage of the fish, and drew attention to the denaturation phenomenon. In the present paper the effect of different types of glazes on the denaturation of proteins is studied by determining their solubility behavior in solutions of high and low ionic strength, ATP-ase activity of muscle brei, and the contractility of muscle fiber in the presence of ATP. ATP-ase activity and the contractility of muscle fiber are the properties associated with the actomyosin fraction of the muscle proteins.

MATERIALS AND METHODS

The following fish—pomphret (Stromateus cinerius), surmai (Cybium commersonii), and mackerel (Scombermicro lepidotus)—selected for the experiment, were frozen after the rigor mortis stage (Nikkila and Linko, 1954).

Pomphrets were quick-frozen at −30°F (−34.44°C) for 4 hours. They were divided in five groups. One lot was dipped in 0.1% ascorbic acid solution for 2 minutes. The remaining fish were divided into 4 groups. One was kept as control, two were glazed with cold water and with solution containing each of 0.5% sodium chloride and glucose, and the fourth group was placed in a gunny sack and dipped in the sodium chloride-glucose solution. The fish in all the groups were stored 9 months at 5°F (−15°C).

Surmai were quick-frozen at −20°F (−28.9°C) for 4 hours. They were then divided into 4 groups; one was maintained as control and the others were glazed with 0.1% ascorbic acid, 1% citric acid, and 1% sodium nitrite solutions. The fish were then stored for 4 months at 5°F (−15°C). The reglazing of surmai was done after every 4 weeks.

At regular intervals the samples of fish were taken for analysis. To avoid scatter in results, the myotomes were separated from the myocommata and were used in the preparation of extracts as suggested by Ironside and Love (1956).

Determination of soluble and sarcoplasmic proteins. The soluble proteins were extracted at room temperature by the method of Dyer *et al.* (1950) in 5% sodium chloride solution, whereas the sarcoplasmic proteins were extracted in phosphate buffer of low ionic strength by Seagran's method (1958a). Proteins in these extracts were determined by the biuret method (Snow, 1950a). This method was standardized by the Kjeldahl nitrogen method (Hiller *et al.*, 1948).

Determination of ATP-ase activity. A weighed sample of finely minced muscle was macerated, washed, and made up to volume. ATP-ase activity was determined by the method of Duboiss and Potter (1943). Blank determinations were carried out in which the enzyme or ATP or both were replaced by water. In this way the amount of inorganic phosphorus liberated from ATP by enzymes was estimated by the method of Sterges *et al.* (1950).

Contractility of muscle fiber. The myotomes were removed from fish and shaken with the veronal buffer containing Mg^{++}. The fibers were removed from these myotomes, and the diameter of the fiber was determined under a microscope before and after the addition of ATP. The reaction period of 15 minutes was kept constant throughout the experiment. The ratio of diameters under these two conditions determines the extent of contractility.

RESULTS

The values for the frozen fish were compared with those for the fresh fish. The results for the soluble proteins are in Fig. 1, 2, 3; data on the ATP-ase activity and contractility of fiber are in Table 1.

Soluble Proteins. The extractability of soluble proteins in 5% sodium chloride in the case of fresh pomphret, surmai, and mackerel was respectively 40, 53 and 48 g/100 g of protein. This amount, however, decreased during the frozen storage.

Pomphrets. In the control group of pomphrets, the solubility of the proteins decreased rapidly to nearly 26% during four months of storage, and decreased very slowly up to 9 months of storage. The loss in the solubility of proteins extractable in 5% sodium chloride was, in general, greater in the control than in fish glazed with water or sodium chloride glucose solution, fish placed in a gunny sack, and fish dipped in ascorbic acid solution before freezing. The order of effectiveness in decreasing the loss in extractability of proteins in 5% sodium chloride was: sodium chloride glucose glaze, gunny sack, water glaze, and ascorbic acid glaze. When the glaze has evaporated or when the effectiveness of dip is diminished, as is the case with ascorbic acid after nearly three months of

Table 1. ATP-ase activity of muscle brei and contractility of muscle fiber of fresh and frozen stored surmai and mackerel.

Fish	ATP-ase activity [a]	Ratio of the fiber diameter [b]
Surmai		
Fresh	1338 ± 61.99	1.83 ± 0.26
Frozen	1329 ± 75.44	....
Stored 4 months		
Control	891 ± 24.75	1.33 ± 0.14
Glazed with ascorbic acid	975 ± 23.87	1.43 ± 0.16
Glazed with citric acid	1108 ± 42.05	1.63 ± 0.09
Glazed with sodium nitrite	1049 ± 30.24	1.52 ± 0.18
Mackerel		
Fresh	1065 ± 69.20	1.56 ± 0.15
Frozen	1063 ± 57.11	....
Stored 4 months		
Control	886 ± 23.64	1.42 ± 0.07
Blocked	923 ± 34.10	1.50 ± 0.05

[a] Expressed as μg of P that would have been liberated by the extract containing 100 mg of wet tissue in 15 min incubation at 37°C.

[b] Ratio of diameters of fiber after treating with ATP to diameters without treatment with ATP.

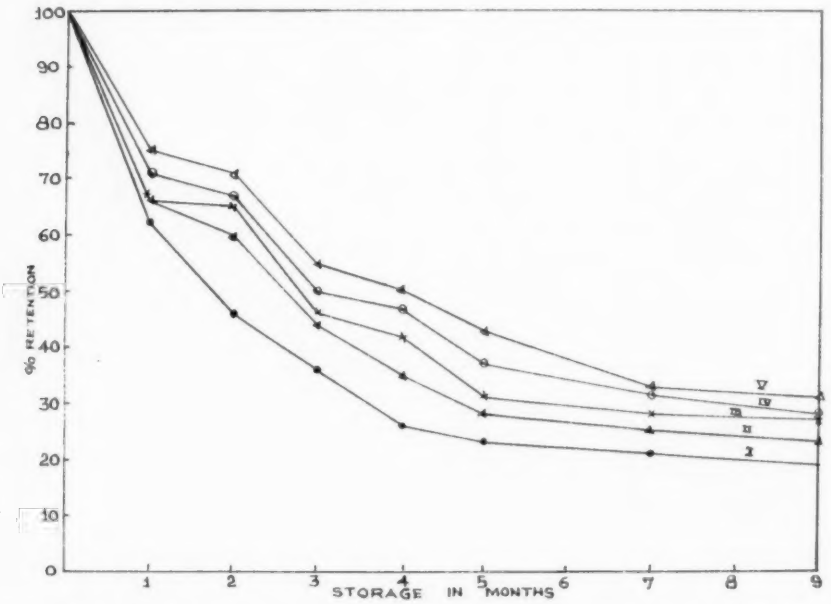

Fig. 1. Effect of frozen storage on soluble proteins of pomphret. From the top down: sodium chloride glucose glaze; gunny sack; water glaze; ascorbic acid dip; control.

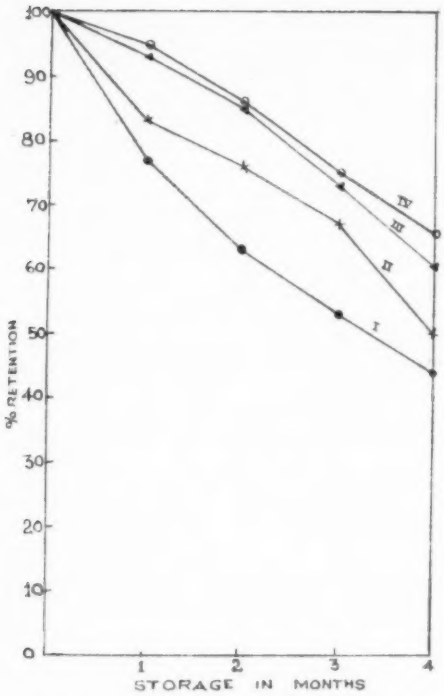

Fig. 2. Effect of frozen storage on soluble proteins of surmai. From the top down: citric acid glaze; sodium nitrite glaze; ascorbic acid glaze; control.

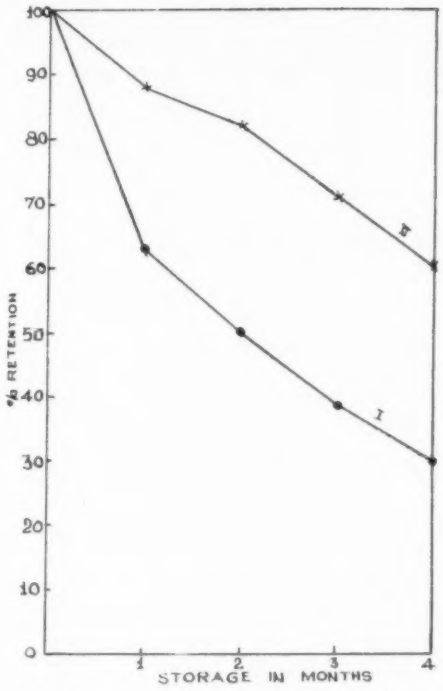

Fig. 3. Effect of frozen storage on soluble proteins of mackerel. Top: block. Bottom: control.

storage, the rate of loss in the extractability of proteins proceeded in a pattern similar to that for the control group, limiting value being reached after 5–6 months of storage.

Surmai. With surmai the extractable proteins in 5% sodium chloride decreased to 44% during 4 months of storage in the control group, whereas the solubility of proteins decreased only to 66% in the group glazed with citric acid and 60% in the group glazed with sodium nitrite. Ascorbic acid glaze was rather less effective.

Mackerels. With mackerel the solubility of proteins in 5% sodium chloride decreased to 31% in the control group in four months of storage, the decrease being faster than in surmai. In mackerels blocked in ice, which served as a permanent glaze during storage, soluble proteins decreased only to 60% during 4 months of storage.

Sarcoplasmic proteins. The level of sarcoplasmic proteins, which constituted 30% of the total soluble proteins, remained practically unchanged during storage of surmai and mackerel. The sarcoplasmic proteins of fresh surmai and mackerels were 15 g/100 g of proteins.

ATP-ase activity and contractility of muscle fiber. After four months of storage ATP-ase activity of the muscle brei of surmai was respectively 66.6, 73.0, 82.8, and 78.4% in the control, ascorbic acid, citric acid, and sodium nitrite groups, as compared to ATP-ase activity of fresh surmai. ATP-ase activity of muscle brei in mackerel after 4 months' storage was 83.3% and 86.2% in the control and blocked mackerels, respectively, as compared to fresh mackerel. Practically no change in ATP-ase activity was noted immediately after the freezing of surmai and mackerels.

Contractility, tested for 15 min in the presence of ATP of the muscle fiber of surmai after 4 months of storage, was respectively 72.7%, 76.4%, 89.1%, and 84.7% in the control, ascorbic acid, citric acid, and sodium nitrite glazes, as compared with fresh muscle fiber. In mackerel the retention of original contractility of fiber was respectively 91.0% and 96.2% in the control and blocked-in-ice groups.

DISCUSSION

The loss in extractability of protein in 5% sodium chloride during storage is widely attributed to the denaturation of proteins (Dyer, 1951; Dyer and Morton, 1956; Dyer *et al.*, 1956; Ironside and Love, 1956; Love, 1956, 1958; Luijpen, 1957; Mahadevan and Carter, 1948; Nikkila and Linko, 1956; Notevarp and Heen, 1940; Snow, 1950b). Love (1958), in discussing denaturation of proteins at low temperatures, suggested that the inorganic salts and other organic compounds that can remain in solution even at very low temperature because of their low eutectic point, come in contact with the protein gel causing denaturation. The fragments of ice try to keep the protein gel away from such solution, but as the storage of frozen fish proceeds, the fragments of ice are used up, developing into big ice crystals and allowing these two components to come in contact with each other. The formation of ice crystals from the fragments is attributed to the dehydration of tissue cells (Dyer, 1951). According to Dyer the proteins present in the sol form in the fresh tissue are converted to gel form, which is denatured by salts in the muscle at the eutectic point. It therefore appears that the dehydration of muscle cells during frozen storage is the main cause favoring the conditions for denaturation. Better retention of soluble proteins in the glazed fish can be explained as follows: The different types of glazes used may prevent dehydration of tissue cells till the glazes are sublimed. This is substantiated in pomphret by the fact that when the glaze is sublimed denaturation proceeds at the same rate as in the control. The trend was the same for surmai glazed with the different solutions and the mackerel kept in the ice block. The denaturation of proteins will be less in the case of a glaze having low water-vapor pressure. This fact resulted in the differences in capacity of preventing the denaturation of protein observed for the different types of glazes. Similar effect of glazes was found by Nikkila and Linko (1956).

Since the mackerel were frozen slowly, denaturation of proteins was faster in the mackerel control group than in the surmai control group; the large ice crystals formed during slow freezing favor denaturation.

That the sarcoplasmic fraction level did not change during frozen storage, confirms Dyer's observation that albumin fraction was not denatured during frozen storage. This shows that the denaturation of proteins is restricted to the actomyosin fraction. This is also in agreement with the findings of Seagran (1958b, 1959), who suggested that the sarcoplasmic fraction is not associated with the origin of drip.

A decrease in ATP-ase activity during frozen storage of fish was shown by Partmann (1954). It is known that ATP-ase activity is associated with the actomyosin fraction, which is the main part of the contractile structure in the muscle. It is surprising that, even though considerable denaturation of actomyosin had occurred, the apyrase activity and the contractility of muscle fiber did not decrease to the same extent. According to Szent-Györgyi (1953) the myosin molecule can be split into two components, the L- and H-meromyosins. H-meromyosin, which constitutes 33% of the total actomyosin, possesses all the ATP-ase activity. During frozen storage L-meromyosin is denatured more than H-meromyosin. The results of the apyrase activity and the contractility of the muscle fiber indicate the extent of stability of the H-fraction.

Acknowledgments

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The Application of Radiation-Distillation to the Production and Isolation of Components of Beef Irradiation Flavor

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(Manuscript received August 4, 1960)

The technique of "concurrent radiation-distillation" (Wertheim *et al.*, 1959) has been applied to the problem of beef irradiation flavor as a means of producing the volatile odor components and isolating them almost immediately after production in quantities large enough to allow chemical identification.

The procedure, which was originally designed for removal of irradiation flavor from milk, had to be modified to allow concurrent radiation-distillation of an aqueous slurry of ground beef and isolation of the relatively large quantities of odorous distillate thus produced.

APPARATUS

The material to be irradiated was circulated in a system (Fig. 1) consisting of an irradiation chamber, a vacuum-maintaining valve, a flash evaporator, a Moyno pump, and a water bath. Water vapor from the flash evaporator, which carries volatile compounds, was condensed in a stainless-steel condenser (cooling surface 28 sq ft), conducted out of the irradiation room through glass tubing and collected in 5-gal. receivers immersed in ice water and protected from light. An efficient vacuum pump (Welch Duo-Seal, Model 1402B) protected by a dry-ice trap, maintained the vacuum desired in the system. De-acrated water (nitrogen washed) was fed into the irradiation chamber in order to maintain a constant volume of liquid in the circulation system. The stainless-steel condenser was cooled with ice water provided from a 55-gal. reservoir by a circulating pump. The irradiation chamber was continuously swept with helium gas in order to maintain an oxygen-free system. The source of radiation was an electron beam produced by a 1-Mev General Electric Resonant Transformer.

Contribution Number 403 from the Department of Nutrition, Food Science and Technology.

Preparation of beef slurry

Raw ground lean beef (10 lb, U. S. Choice, bottom round) was ground and further comminuted in a laboratory-model silent cutter and mixed with an equal volume of water. The resulting slurry was strained through a metal strainer and finally through a double layer of cheesecloth. White fibrous material that did not pass through the cheesecloth amounted to about 13% of the original weight of beef. If this material was not removed from the meat slurry the radiation-distillation apparatus was seriously clogged.

The resulting meat slurry (about 6 L) was diluted to 8 L with water and processed in the concurrent radiation-distillation apparatus.

Preparation of meat distillate

The meat slurry was placed in the irradiation chamber of the apparatus (Fig. 1) and the vacuum pump started. As soon as temperature, pressure, and distillation rate were stable, radiation was started. During the equilibration period condensate was collected in Receiver B. The period required for conditions to become stable varied with the beef slurry being examined. Each sample varied in viscosity and tendency to foam during distillation.

The experimental conditions for a typical preparation of an irradiated beef distillate are given in Table 1.

Table 1. Experimental conditions of a typical preparation of irradiated beef distillate.

Quantity of ground beef	10 lb
Volume of meat slurry	8 L
Dose	5 megarad
Average pressure at pump	23 mm Hg
Average temperature	32–36°C
Rate of evaporation	6 L/hour
Total distillate collected	6–8 L

General Appearance and Odor of Meat Slurries and Distillates

No apparent change in the consistency of the meat slurry was caused by irradiation. Its color

was red-brown before irradiation, and green after. The distillate (pH 5), a slightly cloudy colorless aqueous solution, possessed a very characteristic odor. This odor was determined to be characteristic of irradiated raw beef by simple comparison between the odor of samples of irradiated meat slurries and the odor of slices of irradiated beef. The dose in each case was 2×10^6 rad. It was the informal opinion of eight of nine persons asked, that the odor of the meat slices was less acceptable. However, after these same slices were slurried and strained through cheesecloth in the manner described for the preparation of meat slurries, six persons out of eight felt that these samples, though less disagreeable, were very similar in odor to the original irradiated slurries. These observations indicate the possibility either that the disagreeable quality of the meat slices had been diminished by dilution when slurried, or that the fibrous material removed during straining of the slurry through cheesecloth had contributed the off-odors noted.

Removal of fibrous material caused no diminution of the irradiation odor, since distillates obtained by lyophilization of slurries of beef from which fibrous material had been removed exhibited the typical odor as strongly as did slurries that had not been strained.

It was thus concluded that the odor of the distillate obtained by irradiation of meat slurries was the same as that of irradiated beef slurries themselves, and that this odor closely approximated the odor of irradiated sliced beef after it had been slurried in the manner required for processing by radiation-distillation. It is thus believed that the odor produced by the volatile compounds found in the distillate is typical of irradiated raw beef. Detailed chemical investigation (Wick *et al.*, 1960) of such distillates is therefore justified.

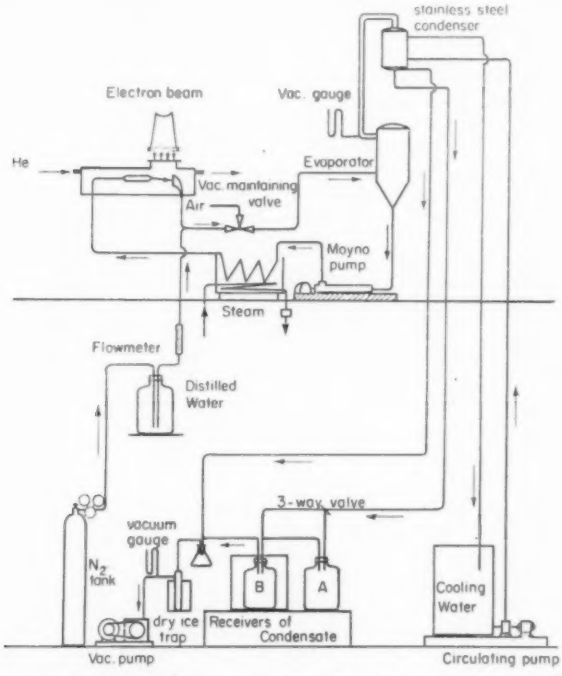

Fig. 1. Concurrent radiation-distillation apparatus adapted for collection of volatile components.

On the basis of the above observations it is believed that concurrent radiation-distillation may be usefully applied to study of the nature of the volatile components of beef irradiation flavor.

Acknowledgments

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This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, QM Research and Engineering Command, U. S. Army, and has been assigned number 2037 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

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Comparative Studies on Media for Counting Anaerobic Bacterial Spores. II.

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(Manuscript received May 18, 1960)

SUMMARY

Subculture counts using 16 different media representing three general types (fresh-meat infusion, formulated media, and dehydrated) were compared for the recovery of severely heated spores of two strains of PA3679. Comparative tests with these same media were made on the recovery of heated spores of PA3679, which had been produced in four sporogenic media. In general, the subculture media prepared from fresh-meat infusions showed the highest recovery counts for all spore suspensions in all experiments. Dehydrated media showed the poorest recovery. The intermediate counts with the formulated media overlapped the counts with the other two groups of media. Pork-pea infusion extract and beef infusion extract consistently yielded the highest counts and were appreciably higher than any of the dehydrated media. Differences were noted between the response of the two strains of PA3679 to the various subculture media. In addition, differences were noted between the responses to various subculture media on a single strain when the spores were grown on different substrates. Spores of a single strain of PA3679 grown in different substrates showed marked differences in heat resistance.

The first paper (Wheaton *et al.*, 1959) under the present title was presented at the 1958 annual meeting of the Institute of Food Technologists. It gives the background for comparison of results on media used by some 26 university, government, and industry laboratories for recovery and counting of anaerobic spores. The 11 media most directly applied to counting techniques by these leaders in heat-resistance studies were used in this laboratory for comparative recoveries of thermally processed and of gamma-irradiated spores of the putrefactive anaerobe, strain PA3679, and two Type B strains of *Clostridium botulinum*. The strain of PA3679 used in the work was the stock-culture organism of this laboratory. When severely heated spores were subcultured in 10 of the media, recoveries were significantly different; counts were highest with media Nos. 3 and 6, beef infusion extract and pork-pea infusion extract.

Work in this same general field has been published by Frank and Campbell (1955) and Riemann (1957). The first authors also worked with a single strain of PA3679 and studied the thermal resistance values of the spores in various recovery media. Riemann reported a similar study on 6 media, involving both *Cl. botulinum* and PA3679. His media were generally dissimilar to the ten used in the first work at this laboratory. Campbell and Frank (1956) also reported a study of the nutritional requirements of putrefactive anaerobes. Included in that work were 10 strains of PA3679 that represented cultures from widely separated laboratories. Consequently, the collection included variations in environmental growth conditions in maintaining the cultures over varying periods of years. From the data, it appeared that certain growth factors might be required by some strains but not by others.

This observation suggested that the present work should be based on strains of PA3679 from other laboratories to compare their ability to initiate outgrowth on various media with that of the stock strain of this laboratory as already reported in the first publication (Wheaton *et al.*, 1959). To broaden the coverage of media, six additional media suggested in 1957 were added to the ten employed with heat-processed spores of PA3679. One of the new PA3679 strains was used to study the effect of the

medium employed to produce the spores upon the outgrowth of the germinating spore in the widely different types of recovery media.

Continuing the form used in the previous publication, the separate phases of the current work are summarized as follows:

Phase IV. Determination of the effect of 16 different media on the recovery of severely heated spores of PA3679. The strain used, designated the IFT strain, was supplied by Professor W. B. Esselen.

Phase V. Determination of the effect of the same 16 media on the recovery of severely heated spores, using a strain of PA3679 designated No. 42 and furnished by Dr. C. F. Schmidt.

Phase VI. Determination of the effect of producing the spore suspensions in 4 different sporogenic media. The IFT strain of PA3679 was used for the 4 spore suspensions, and recovery counts were made in all 16 media as for Phases IV and V.

MATERIALS AND METHODS

Organisms. *Phase IV.* The IFT spore suspension supplied by Prof. Esselen in 1957 has been grown in a pork infusion broth described by Reed *et al.* (1951). The inoculated medium was incubated 1 week at 98°F (37°C), followed by 2 weeks of incubation at 86°F (30°C). After centrifuging, the spores were stored at 34–36°F (1–2°C) in sterile tap water without any washing.

Phase V. Strain No. 42 was grown in Difco egg meat medium (10 g to 150 ml water) for 10 days at 85°F (29°C). After centrifuging, the spores were stored in their natural liquor.

Phase VI. The 4 suspensions of the IFT strain used for this experiment were prepared from the original suspension as used for the Phase IV experiment. Through the cooperation of the University of Massachusetts and the National Canners Association, it was possible to secure spores grown on media regularly used by these laboratories and handled in accord with their experience. Dr. Hamed M. El-Bisi, of the University of Massachusetts, prepared two of the suspensions, one grown in pork infusion broth and the other in trypticase medium. The pork medium was that of Reed *et al.*, as used for the Phase IV suspension. The other spore crop was produced at 86°F (30°C) in a trypticase medium of Dr. El-Bisi's own formulation until maximum sporulation was obtained (72 hours). The spore crops were harvested and washed 3 times in cold sterile distilled water. Mr. J. Yesair, of the National Canners Association, prepared a third suspension in beef liver extract medium. The other ingredients were tryptone, soluble starch, sodium thioglycollate, and dipotassium phosphate, and the pH adjusted to 7.18. The inoculated medium was incubated 4 days at 98°F (37°C), followed by 15 days at 80°F (26°C). After centrifuging, the spore sediment was washed twice with sterile tap water.

The fourth spore suspension was prepared at this laboratory in a beef-heart infusion medium according to the following formula: beef-heart infusion, 1000 ml; gelatin, 10 g; tryptone, 10 g; isoelectric casein, 5 g; glucose, 0.3 g; Na_2HPO_4, 4 g; and sodium citrate, 3 g. After addition of the

Table 1. Media employed for spore recovery.

Medium no.	Agar medium	$NaHCO_3$	Sterilization (min/°F)	Tube stratification	Incubation (°F)
1	Peptone colloid	None	15/250	None	98
2	Eugonagar	.5 ml—10%	15/243	None	98
3	Beef infusion	None	20/250	Yes	86
4	Tryptone yeast extract	.2 ml—10%	15/250	Yes	86
5	Pork infusion	None	20/250	Yes	86
6	Pork-pea infusion	.4 ml—5%	12/250	Yes	86
7	Thioglycollate w/o glucose	None	15/250	None	98
8	Brain-heart infusion with PAB	None	15/250	None	98
9	T BEST	None	15/250	None	86
10	Beef extract	.2 ml—10%	15/250	Yes	98
11	Beef-liver infusion	.5 ml—5%	15/250	Yes	86
12	Yeast extract medium	.3 ml—5%	15/250	Yes	
13	Brain-heart infusion	Yes–no	15/250		86
14	AC medium		15/250		
15	Beef-heart infusion	None	20/250	Yes	90
16	Modified Brewer's	None	15/250	None	98

first two ingredients to the infusion, pH was adjusted to about 8.5 and the isoelectric casein added. After the casein had dissolved, the other ingredients were added and pH was adjusted to 7.2. The medium was distributed in flasks with a small amount of the dried meat, and sterilized. After inoculation, the flasks were incubated 12 days at 86°F (30°C), and the medium filtered through cheese cloth and cotton, and centrifuged once. After decanting the liquid phase, the spores were resuspended in a small volume of the mother liquor.

It was found necessary to concentrate all spore suspensions except those from the beef heart by centrifuging in order to get a suitable number of spores surviving the thermal process of 2 minutes, 30 seconds at 250°F (121°C) in the dilutions used. The dilutions selected for processing and culturing are described in the ensuing section. The approximate ages of the spore suspensions when the experimental runs were started were: beef-liver suspension, 66 days; beef-heart suspension, 73 days; trypticase suspension, 60 days; pork infusion suspension, 46 days.

Thermal processing. All spores subjected to heating were suspended in phosphate buffer (pH 7.0). Depending on the experiment, .01, .02, or .03 ml was added to the standard cups and aluminum boats used in the thermoresistometer described in the previous paper.

The processed spores were collected in sterile water after passage through the sterilizing chamber. The volume of water used for collecting the processed spores varied with the experiment. For each of the three runs with the IFT strain shown in Table 2, six .01-ml portions of a 1:5 dilution of the suspension were heated in the six slots and collected in 6 tubes, each containing 20 ml of distilled water. The contents of these tubes were pooled, and the composite sample subcultured.

In Table 3, which presents the results with strain No. 42, six .03-ml portions of a 1:4 dilution of the spore suspension were placed in 6 cups and processed, after which the heated samples were collected in 6 tubes, each containing 15 ml sterile water. The water from the 6 tubes was pooled, and this material used for culturing.

Unlike the procedures for Phases IV and V, the spore dilutions employed in Phase VI, as well as the volumes of collection water for the heated spores, had to be varied. Surviving spores after processing had to be of such an order that the most favorable media would not result in tubes that were uncountable because of the density of organisms. This permitted the use of only one cup per

Table 2. Recovery counts of thermally processed spores[a] of PA3679 (IFT strain).

Medium	Average[b] of five 1-ml portions			Av.[b] of all 3 experiments
	Expt 1 (6/30/59)	Expt 2 (7/7/59)	Expt 3 (9/2/59)	
Fresh-meat infusions				
3. Beef-inf. ext.	37.8	28.2	39.4	34.7
6. Pork-pea inf. ext.	12.2	13.1	25.9	16.1
15. Beef-heart inf. ext.	11.5	11.4	13.4	12.1
5. Pork inf. ext.	6.1	9.3	10.1	7.8
11. Liver inf. ext.	1.7	3.2	6.0	3.2
Formulated media				
9. T BEST	9.2	6.9	7.6	7.8
12. Yeast ext.	7.1	5.2	7.7	6.5
10. Beef ext.	4.4	4.9	6.8	5.3
4. Tryptone yeast ext.	1 colony in 15 tubes			.07
16. Modified Brewer's	15 tubes negative			0
Dehydrated media				
8. Brain-heart with PAB	1 colony in 15 tubes			.07
1. Peptone colloid agar	15 tubes negative			0
2. Eugonagar	15 tubes negative			0
7. Thioglycollate agar w/o glucose	15 tubes negative			0
13. Brain-heart w/sol. starch + agar	15 tubes negative			0
14. AC medium + agar	15 tubes negative			0

[a] .01-ml portions of 1:5 dilution processed 3 minutes at 250°F. Initial spore counts for experiments 1 and 2 were 340,000 and 360,000 per .01 ml, respectively, whereas no determination was made for experiment 3.

[b] Geometric means.

Table 3. Recovery counts of thermally processed spores [a] of PA3679 (No. 42 strain).

Medium	Av.[b] of five 1-ml portions			Av.[b] of all 3 experiments
	Expt 4 (8/25/59)	Expt 5 (8/31/59)	Expt 6 (9/8/59)	
Fresh-meat infusions				
6. Pork-pea inf. ext.	13.7	7.7	15.7	11.8
3. Beef inf. ext.	14.3	7.9	12.3	11.2
15. Beef-heart inf. ext.	2.8	1.7	2.0	2.1
5. Pork-inf. ext.	1.8	1.7	1.6	1.7
11. Liver inf. ext.	0.8	no colonies	5.9	1.3
Formulated media				
12. Yeast ext.	2.7	2.4	2.7	2.6
9. T BEST	0.8	1.0	1.1	1.0
10. Beef ext.	0.6	2.7	0.6	0.8
4. Tryptone yeast ext.	1.0	1.0	0.8	0.9
16. Modified Brewer's	2 colonies in 15 tubes			0.1
Dehydrated media				
8. Brain-heart with PAB	5 colonies in 15 tubes			0.3
13. Brain-heart w/sol. starch + agar	4 colonies in 15 tubes			0.3
14. A.C. medium + agar	4 colonies in 15 tubes			0.3
1. Peptone colloid agar	3 colonies in 15 tubes			0.2
2. Eugonagar	15 tubes negative			0
7. Thioglycollate agar w/o glucose	15 tubes negative			0

[a] 0.3-ml portions of 1:4 dilution processed 2 minutes, 24 seconds at 250°F. No initial spore counts made.
[b] Geometric mean.

slot in the thermoresistometer equipment, and was carried out with the following dilutions, etc.:

Beef-heart suspension. One one-hundredth ml of a 1:20 dilution was processed, and the heated cup was collected in 20 ml of water from which a 1:25 dilution was made in distilled water. Sixteen 1-ml portions were transferred from this last dilution to Veillon tubes, designating by number a tube for each medium.

Beef-liver suspension. One one-hundredth ml of a 1:5 dilution was processed, the heated cup collected in 20 ml of water, and sixteen 1-ml portions distributed among 16 Veillon tubes as above.

Pork infusion suspension. One one-hundredth of a 1:5 dilution was processed and collected in 25 ml of water, from which a 1:5 dilution was made, and the required sixteen 1-ml portions were removed.

Trypticase suspension. Two one-hundredths of a 1:5 dilution was processed and collected in 20 ml of water, from which the sixteen 1-ml portions were transferred to the Veillon tubes.

Media. Both dehydrated media and media formulated from individual ingredients were included in the study. Since it is of interest to know their composition, all non-dehydrated formulae are given in detail below. The commercial names for the dehydrated products are also listed.

A) Fresh-meat infusions

3. *Beef infusion extract*
   1000 ml beef infusion
   5 g peptone (Difco)
   1.6 g tryptone (Difco)
   1.0 g glucose (Merck)
   1.0 g soluble starch (BBL)
   1.25 g K_2HPO_4
   2.0 g sodium thioglycollate (BBL)
   15.0 g agar (Difco)
   pH 7.4
5. *Pork infusion extract*
   1000 ml pork infusion
   5 g peptone (Difco)
   1.5 tryptone (Difco)
   1.25 g K_2HPO_4
   1 g glucose
   1 g sodium thioglycollate
   1 g soluble starch
   15 g agar
   pH 7.2–7.4
6. *Pork-pea infusion extract*
   800 ml pork infusion
   200 ml pea infusion
   5 g peptone
   1.6 g tryptone

1.25 g K_2HPO_4
1 g soluble starch
0.5 g sodium thioglycollate
15 g agar
pH 7.0

11. *Liver infusion extract*
1000 ml beef liver infusion
10 g tryptone (Difco)
1 g soluble starch
1 g K_2HPO_4
0.5 g sodium thioglycollate (Difco)
15 g agar
pH 7.1

15. *Beef-heart infusion extract*
1000 ml beef heart infusion
1 g soluble starch
5 g glucose
10 g tryptone
1.25 g K_2HPO_4
1 g yeast extract
15 g agar
pH 7.0

B) Formulated media

4. *Tryptone yeast extract*
1000 ml water
15 g tryptone (Difco)
5 g yeast extract
1 g sodium thioglycollate
15 g agar
pH 7.4

9. *T best*
1000 ml water
10 g tryptone
1 g glucose
1 g yeast extract
1 g soluble starch
1.25 g K_2HPO_4
3 g beef extract
0.5 g sodium thioglycollate
16 g agar
pH 7.2

16. *Modified Brewer's*
1000 ml water
10 g proteose peptone No. 3 (Difco)
5 g yeast extract (Difco)
5 g tryptone
10 g glucose
5 g NaCl
1 g soluble starch (Difco)
2 g sodium thioglycollate
.003 g resazurin
1 g agar
pH 7.2

10. *Beef extract*
1000 ml water
4 g beef extract
5 g peptone
1.5 g tryptone
1.25 g K_2HPO_4
2 g soluble starch
3 g yeast extract
0.25 g ascorbic acid
15 g agar
pH 7.4

12. *Yeast extract*
1000 ml water
10 g yeast extract (Difco)
5 g thioglycollate supplement (BBL)
1 g soluble starch (Difco)
2 g K_2HPO_4
15 g agar
pH 7.3

C) Dehydrated media

1. Peptone colloid agar
2. Eugonagar
7. Thioglycollate agar without glucose (Difco).
8. Brain-heart with *p*-amino-benzoic acid and 0.35% agar (Difco)
13. Brain-heart infusion with 0.1% soluble starch and 1.5% agar
14. AC medium with 1.5% agar

The variations in preparation and use of these different media are summarized in Table 1.

Culturing. Veillon tubes (12 mm diameter and 20–22 cm long) made of Pyrex glass tubing were used for all counts. The experiments were performed in randomized blocks of 16 tubes, each representing a test medium. One-ml samples of the water containing the heated spores were transferred to the coded tubes, some of which contained $NaHCO_3$. Each medium, after cooling to 108–111°F (42–44°C), was added to the inoculated tubes in random order, and after solidification a layer of the stratification (paraffin-Vaseline mixture or agar with thioglycollate) material was added where stipulated (Table 1). All tubes were incubated 1 wk at 86°F (30°C) unless they became uncountable sooner, because of gas and turbidity.

Counting. Colonies developing from surviving spores were counted on a modified Quebec colony counter on which had been placed an adapter for the Veillon tubes.

RESULTS AND DISCUSSION

General observations. The results of the three phases of the current study involving severely heated spores are shown in Tables 2, 3, 4, 5, and 6. As expected, gas formation occurred in the culture tubes containing the

Table 4. Recovery counts of spores of PA3679 (IFT strain) from 1-ml portions of four spore suspensions.[a]

Media	Expt 7[b]/Expt 8[c]/Expt 9[d]			
	Beef	Liver	Pork	Trypticase
1	0/ 0/ 0	1/ 0/ 1	0/ 0/ 0	0/ 0/ 0
2	4/ 2/ 0	2/ 0/ 1	1/ 0/ 1	0/ 0/ 0
3	63/56/72	60/22/23	40/18/36	7/12/25
4	35/18/35	13/ 5/12	0/ 2/ 6	3/ 0/ 1
5	48/45/57	39/21/25	17/27/16	2/ 3/12
6	82/72/85	79/31/27	30/21/60	18/26/64
7	5/ 3/ 3	4/ 0/ 2	0/ 0/ 0	1/ 0/ 0
8	0/ 0/ 1	0/ 0/ 0	0/ 1/ 2	0/ 0/ 0
9	35/32/33	13/ 8/16	9/17/22	3/ 4/ 6
10	38/31/35	18/10/12	17/24/15	5/11/17
11	14/16/20	14/ 3/15	6/ 4/ 5	3/ 0/11
12	35/34/43	32/ 4/ 9	6/ 7/10	3/ 0/10
13	2/ 2/ 3	0/ 0/ 2	1/ 2/ 2	0/ 0/ 7
14	6/ 3/ 6	0/ 2/ 0	4/ 2/ 2	0/ 0/13
15	22/12/23	24/ 7/ 9	14/18/16	2/ 2/21
16	2/ 1/ 5	0/ 0/ 2	0/ 0/ 0	0/ 0/ 6

[a] All spores processed 2½ minutes at 250°F.
[b] Initial spore counts: beef, 840,000; liver, 86,000; pork, 580,000; trypticase, 7,360,000.
[c] No initial spore counts.
[d] Initial spore counts: beef, 1,300,000; liver, 108,000; pork, 540,000; trypticase, 9,000,000.

Table 6. Comparative resistance of PA3679 (IFT strain) spores[a] produced in four media.

Spore production medium	Av. initial spore count	Av. survival count[b]	% survival
Beef heart	1,000,000	5,500	550×10^{-3}
Liver	95,000	96	100×10^{-3}
Pork	560,000	550	100×10^{-3}
Trypticase	8,100,000	48	0.6×10^{-3}

[a] Spores processed 2½ minutes at 250°F.
[b] Geometric mean of counts in all media except No. 1.

media with glucose at or soon after the 48-hour incubation period. These same media in general appeared to produce larger colonies than the sugar-free formulations. In some instances it is possible that the counts secured with the sugar-containing media might be somewhat low because of gas formation, but the errors are considered of low magnitude. As reported previously, 72 hours appears to be the incubation period that gives near-maximum count. It was also noted that evidence of growth appeared more quickly in the sugar-containing media than in the sugar-free media.

Table 5. Summary table of recovery counts of thermally processed spores[a] of PA3679 (IFT strain) produced in four media.

Subculture medium	Average[b] counts per ml of three expts				
	Spores produced				Av.[b] of all 4 suspensions
	in beef	in liver	in pork	in trypticase	
Fresh-meat infusions					
6. Pork-pea inf. ext.	78	40	33	32	43
3. Beef inf. ext.	62	31	29	13	29
5. Pork inf. ext.	49	27	19	4.1	18
15. Beef-heart inf. ext.	18	11	16	4.4	11
11. Liver inf. ext.	16	8.4	4.8	2.5	6.4
Formulated media					
10. Beef ext.	34	13	18	9.7	17
9. T BEST	33	12	15	4.1	12
12. Yeast ext.	37	10	7.4	2.4	9.1
4. Tryptone yeast ext.	28	9.0	1.8	1.1	4.8
16. Modified Brewer's	2.1	0.8	no colonies	1.1	1.0
Dehydrated media					
14. A.C. medium + agar	4.7	0.8	2.5	1.5	1.9
13. Brain-heart w/sol. star. + agar	2.3	0.8	1.6	1.2	1.4
7. Thioglycollate agar w/o gluc.	3.5	1.6	no colonies		1.1
2. Eugonagar	1.6	1.0	0.8	no colonies	0.9
8. Brain-heart w/PAB	0.6	no colonies	1.0	no colonies	0.6

[a] Spores processed 2½ minutes at 250°F.
[b] Geometric mean.

It should be noted in Table 4, giving results with the 4 spore suspensions, that a wide range of spores were used in the initial dilution of the suspensions produced in the 4 different sporogenic media. This particularly applies to the trypticase-produced spores, where the initial concentration of spores was in the range of $7–9 \times 10^6$. While this work made no effort to include a study of thermal resistance of spores produced in various menstrua, pronounced differences in resistance became apparent. A further investigation will be made of this particular aspect, specifically by ascertaining the spore destruction rates and the possible role of dipicolinic acid content of the spores of the respective suspensions. Table 6 presents initial counts, survival counts, and per cent survival of spores grown in each of the 4 menstrua.

Statistical analysis. *Phase IV and V.* Three-factor analysis of variance (Snedecor, 1956) was performed on log transformations of 240 individual tube counts for both organisms and media No. 3, 5, 6, 9, 10, 11, 12, and 15. The other media showed mostly negative tubes. The occasional negative tubes in the data analyzed were arbitrarily taken as ½ organism. The analysis is shown in Table 7A. The conclusions drawn were as follows:

1) Comparisons of the different subculture media were not quantitatively consistent in all different experiments (significant at 1% level).

2) The two organisms (the IFT strain, and strain 42) responded differently to the various media (significant at 5% level).

3) In spite of the above, however, some media showed significantly higher counts than others with both strains used in Phases IV and V. The fresh-meat infusions gave better results as a group, with the beef infusion and pork-pea infusion showing the highest counts.

Phase VI. Three-factor analysis of variance was performed on the log transformations of 180 subculture counts of spores grown in four substrates and counted in 15 subculture media (all media were represented except No. 1, which showed almost no recovery). The analysis of variance is presented in Table 7B. The conclusions were as follows:

Table 7A. Analysis of variance—Phase IV and V.

Source of variation	Degrees of freedom	Mean square
Organism	1	19.827
Subculture medium	7	3.966**
Experiment within organism	4	0.252
Organism × medium	7	0.472*
Medium × expt within organism	28	0.177**
Replicate counts	192	0.048
Total	239	

Table 7B. Analysis of variance—Phase VI.

Source of variation	Degrees of freedom	Mean square	
Growth substrate	3	3.459	
Subculture medium	14	4.435	
Replication	2	1.113	
Growth × subculture medium	42	0.141**	
Growth × rep.	6	0.392**	
Subculture medium × rep.	28	0.069	.062
Growth × subculture medium × rep.	84	0.060	
Total	179		

* Significant at 5% level.
** Significant at 1% level.

1) The subculture media performed consistently in the three experiments although the survival of spores produced in the various media showed differences in survival in different experiments (significant at 1% level).

2) Spores produced in different media responded differently to the various subculture media (significant at 1% level).

3) In spite of the above, some media showed consistently higher counts than others for all four spore suspensions and all three experimental runs (significant at 1% level). These were beef infusion and pork-pea infusion.

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Spectral Measurements of Hematin Pigments of Cooked and Cured Meats

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SUMMARY

Reflectance spectra, low temperature absorption spectra and difference absorption spectra are used for the identification of the hematin pigments of cooked and cooked cured meats. These spectra provide more critical identification of the cooked and cured meats as hemochromes.

Visible spectra provide valuable information for characterization of cooked meat pigments, and for measurements of changes during processing and storage (Brown and Tappel, 1958a, b; Naughton *et al.*, 1958). Yet there are few spectral studies of the hematin pigments of cooked meat and cooked cured meat because these pigments give diffuse bands in reflectance spectra and do not allow conventional absorbance spectra because of insolubility. The pigments of cooked meats and cooked cured meats have been characterized by reflectance spectra as denatured globin hemochromes and denatured globin nitric oxide hemochromes, respectively (Tappel, 1957a,b). A major difficulty in this characterization was the diffuse bands in the reflectance spectra. Progress in determining the spectral characteristics of these insoluble pigments requires both new experimental techniques and the best spectrophotometers available.

This paper describes the use of reflectance spectra, low-temperature absorbance spectra, and difference absorption spectra for studies of the hematin pigments of cooked and cooked cured meats.

EXPERIMENTAL

Reflectance spectra were recorded with a Beckman DK-2 spectrophotometer equipped with reflectance attachment. Low-temperature spectra were recorded using the apparatus and techniques described by Estabrook (1956) and the split-beam spectrophotometer described by Chance (1954). The meat was ground with equal volumes of glycerol to give a suspension containing 50% glycerol. Temperature of −190°C was maintained with liquid nitrogen. The monochrometer was calibrated with cytochrome C. Half band widths were about 0.5 mμ. Plexiglas cuvettes with optical path of 3 mm were used. This method has been previously used for measuring low-temperature spectra of cytochromes in solution (Estabrook, 1956), in mitochondria (Estabrook and Mackler, 1957), and in muscle. Difference spectra were recorded at room temperature in 1-cm cells using the split-beam spectrophotometer. Meat was homogenized in 50% glycerol to keep the sample suspended and to reduce light scattering. The main usefulness of difference spectra is that the light scattering of the insoluble pigments is canceled out (Chance, 1953, 1954). In practice the sample cuvette contains pigment in the reduced state and the reference cuvette contains pigment in the oxidized state. Meat pigments were reduced with sodium hydrosulfite and oxidized with potassium ferricyanide.

RESULTS AND DISCUSSION

Fig. 1 shows the reflectance spectra of cooked cured ham obtained with the Beck-

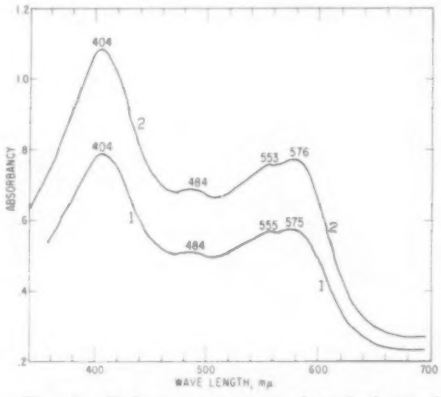

Fig. 1. Reflectance spectra of cooked cured ham: 1) light muscle; 2) dark muscle.

man DK-2 spectrophotometer. This instrument gives superior resolution over the DR spectrophotometer previously used in our identification of cooked meat pigments. The wavelengths of minimum reflection (maximum absorbancy) are shown on top of the two curves. These show good agreement with those previously obtained and confirm the characterization of this pigment as a hemochrome. These reflectance minima, in the regions 575 mμ for the α peak, 553 mμ for the β peak, a 484 mμ peak, and 405 mμ for the γ peak, are characteristic of cooked cured meats.

Figures 2 and 3 show low-temperature absorbance spectra of cooked cured meats. Low-temperature spectra are the most selective method for characterization of hemochromes, and have become a method of choice for differentiating cytochromes in naturally occurring mixtures. The main advantages are peak sharpening and intensification, especially of the α peaks, because of multiple reflection within the sample from the micro ice crystals. This peak sharpening and intensification are seen here for the α, β, and 483 peaks. In these low-temperature spectra one can see the shift of the α and β peaks to shorter wavelengths than those in reflectance spectra. The α peaks shift from 576 to 568, and β peaks shift from 553 to 547. Similar shifts to shorter wavelengths have been observed for the cytochromes a, b, and c, although the shifts are less (Estabrook and Mackler, 1957). Little shift of the γ peak would be expected, so it should be in the 405 mμ region. The 412 γ peak for

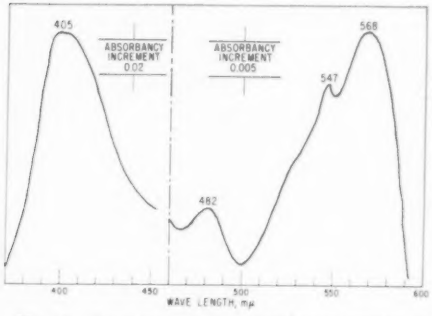

Fig. 2. Low-temperature absorbance spectra of cooked cured ham.

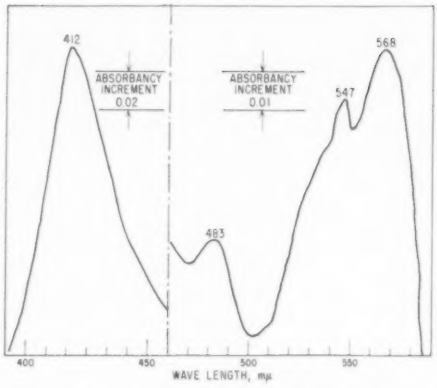

Fig. 3. Low-temperature absorbance spectra of bologna.

bologna is unusual, and cannot be explained at present.

Besides defining low-temperature spectra for cooked cured meats, these data allow more positive assignment of these meat pigments as hemochromes. They give more positive evidence for the occurrence of the 482–3 mμ peak, a peak characteristic of cured meat pigments but not of other hemochromes.

Fig. 4 gives typical difference spectra obtained with cooked beef pigment in the reduced state minus cooked beef pigment in the oxidized state. Compared to previous reflectance spectra of these pigments (Tappel, 1957a), the different spectra have very sharp bands. Since the different spectra are spectra of reduced pigment minus the oxidized pigment, its characteristic peaks are usually close to those of the reduced pigment but not identical. In the case of cooked beef, the difference spectra give peaks at 550, 519, and 419 mμ, whereas the minima in the reflectance spectra for the reduced pigment are 563, 535, and 422 mμ. The shift in the α and β peaks to shorter wavelengths in difference spectra is caused by the subtraction of the 547 α peak of cooked beef in the oxidized state. These sharp bands and their relative intensities allow good characterization of the cooked meat pigments in the reduced state as typical hemochromes. In fact, this different spectrum is almost exactly like that of cytochrome c, which has α, β, and γ peaks at 550, 520, and 418 mμ, respectively.

The cooked-beef pigment in the reduced state forms a carbon monoxide derivative, which gave a reduced carbon monoxide minus oxidized spectrum of 557 mμ as the α maximum, 532 mμ as the β maximum, 406 mμ as the γ maximum, and 427 mμ as the γ minimum. Here again the formation of sharp γ maximum and minimum is very characteristic of carbon monoxide hemochromes and a good method of characterizing these hemochromes. The carbon monoxide derivative of cytochrome a_3 has been most studied (Chance, 1953). The reflectance spectra of the carbon monoxide derivative of the cooked-beef pigment has been studied. As with all difference spectra, the α and β peaks are not in the same position as those for an absolute absorbance or reflectance spectrum.

Fig. 5 shows difference spectra of reduced nitrited beef minus oxidized cooked beef. This spectrum gives an example of the definition that can be obtained with cooked cured meats. In this study, it was difficult to resolve the α and β peaks. Here the β peak merges with the α peak and shows up as a shoulder at 548, with the α peak at 563 mμ. However, the peak in the 480 region, here at 474 mμ, is well defined. All of these peaks are displaced to shorter wavelengths than those in reflectance spectra.

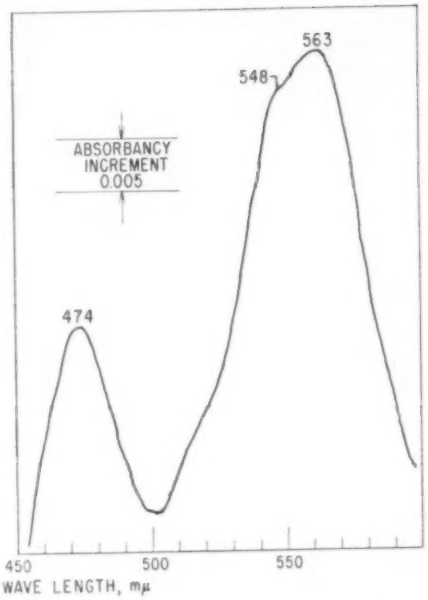

Fig. 5. Difference spectra of nitrited cooked beef.

Acknowledgments

Part of this research was performed while the author was on sabbatical leave at the Johnson Foundation for Medical Physics, University of Pennsylvania. Professor Britton Chance kindly provided use of the spectrophotometers and other facilities of the foundation.

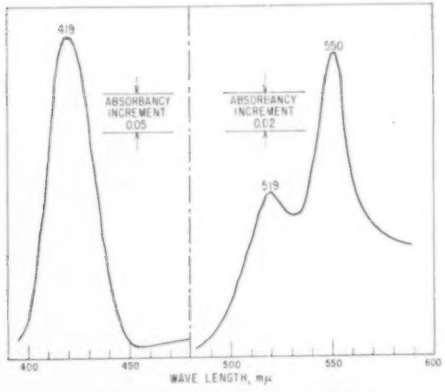

Fig. 4. Difference spectra of cooked beef.

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Fatty Acids of the Lipids of Vegetables. I. Peas *(Pisum sativum)*[a]

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(Manuscript received September 10, 1960)

SUMMARY

Changes taking place in the fatty acids of the lipid material of the edible pea were investigated. Peas were held 1 year in storage at −17.8°C in the raw and enzyme-inactivated condition, to determine changes taking place in the fatty acids. Under these conditions, all of the several fatty acids in the phospholipid fraction showed large losses in the raw as contrasted with the enzyme-inactivated samples. At the same time the neutral fats in the same sample showed losses for all the unsaturated fatty acids in the raw when contrasted with those extracted from the enzyme-inactivated material. The fatty acids in the neutral fat and the free fatty acids of the raw material showed a net gain in the total quantity of palmitic acid. It seems possible that this increase came from the three unsaturated C_{18} acids.

The biochemistry of the developing pea *(Pisum sativum)* seed involves the presence and alteration of many groups of compounds. Among these, the fatty acids are likely to occupy a place of importance. Information on the nature of these fatty acids is sketchy, if not lacking. It is known (Lee, 1954; Lee and Wagenknecht, 1951) that raw peas held at −17.8°C for extended periods show a decided increase in the acid content of the extracted crude fatty material. The fatty material in fresh peas, extracted with ethyl ether, amounts to about 0.4%. The crude lipid extracted with chloroform-methyl alcohol solvent, however, amounts to about 1.2% of the fresh pea. This amount of lipid is small, but its importance cannot be judged solely by its quantity.

METHODS

Samples of peas, Perfected Freezer variety, were harvested at the stage for use as a fresh vegetable from the Experiment Station plots, were shelled mechanically, and were blanched in boiling water for 1 min to inactivate the enzymes so as to limit changes that would otherwise take place. They were then placed in storage at −17.8°C for a year before lyophilization and extraction of the lipid. The lyophilization was done in a Stokes freeze-dryer, which permits quantity operations. At the same time, other lots of this material, which were in the untreated (raw) condition, were placed in storage at −17.8°C and held for a year. This was done to determine the nature of the changes in the fatty acids that take place in raw peas during storage at −17.8°C.

In each case, the lipid was extracted as follows: The lyophilized material was extracted with 2:1 $CHCl_3$ and MeOH (Wagenknecht, 1957).

The crude lipid was then treated with reagent-grade acetone to separate the acetone-soluble fraction from the acetone-insoluble material. The acetone-soluble fraction is made up largely of fats, free fatty acids (FFA), and unsaponifiable matter. The acetone-insoluble fraction comprises the phospholipids.

Free fatty acids. The FFA were obtained as sodium salts by extracting 3 times the acetone-soluble fraction dissolved in a 1:1 mixture of Et_2O and petroleum ether with 1% Na_2CO_3 (Mattick and Lee, 1959). The pooled sodium soap solutions were acidified and extracted in the usual manner.

Neutral fats. The ethereal layer from the sodium carbonate extraction was taken down in the rotary evaporator. Enough alcoholic potassium hydroxide was added so that each 5 g of the residue was treated with the following solution: 30 ml redistilled EtOH (95%), 1 g KOH, and 5 ml H_2O, and refluxed 3 hours in an atmosphere of nitrogen. The unsaponifiable matter was extracted from the

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final saponified solution as directed by the A.O.A.C. (Assoc. Offic. Agr. Chemists, 1945). The A.O.A.C. directions for saponification were not fully employed because it was deemed advisable to have only a slight excess of alkali rather than the larger excess directed by the A.O.A.C. This would tend to prevent more than a minimum of change during the refluxing.

The fatty acids were released from the aqueous alcoholic layer by acidifying with 10% H_2SO_4 and extracting with a 1:1 mixture of Et_2O and petroleum ether. This fraction constitutes the fatty acids from the neutral fats.

Phospholipids. The acetone-insoluble extract was treated with 30 ml of solvent ($CHCl_3$–MeOH, 2:1) for each 15 g of original crude lipid, and allowed to stand 2 hours at 4°C under nitrogen. It was then filtered off on No. 2 Whatman filter paper. This was repeated 5 more times, and the combined filtrates were stripped of solvent by means of the rotary evaporator. Each 10 g of acetone-insoluble material (phospholipids) was treated with 160 ml of 20% HCl (Wooley, 1943) and simmered under nitrogen for 28 hours. The fatty acids were recovered as usual.

The various lots of fatty acids were methylated by the microtechnique of Roper and Ma (1957). The acids were then identified and determined by gas chromatography.

An Aerograph gas chromatographic instrument, Model A-100, was employed for fractionation, identification, and quantitative determination of the methyl esters of the fatty acids. A 10-ft column packed with Craig polyester succinate (butanediol succinate) (Craig and Murty, 1959) was maintained at 217°C with helium used as the carrier gas at a flow rate of 80 ml a minute. A filament current of 210 ma was maintained on the standard Aerograph four-filament detector. A Varian 10-mv recorder was modified with an Aerograph range-selector switch to give a 1-mv full-scale sensitivity. This gas chromatographic technique has been found accurate within 3% (Craig and Murty, 1959).

RESULTS AND DISCUSSION

From the results (Table 1) it seems that palmitic acid increases at the expense of some of the other fatty acids. It was deemed desirable, therefore, to calculate the results on the basis of palmitic acid. Table 2 shows the changes in the principal (in quantity) fatty acids of the phospholipids, neutral fats, and

Table 1. A comparison of the distribution of fatty acids in extracted pea lipid (mg fatty acids per 1500 g of peas, fresh-weight basis; peas held 1 year at −17.8°C).

	Untreated			Enzyme-inactivated		
Fatty acids	Phospho-lipid	Neutral fat	Free fatty acid	Phospho-lipid	Neutral fat	Free fatty acid
Caprylic			trace			trace
Capric	33.4		trace	25.5		2.9
Lauric						trace
Myristic		trace	73.1		43.7	2.4
Palmitic	603.0	880.4	2563.4	1230.5	641.3	10.5
Stearic			trace	8.5	7.3	trace
Oleic	14.3	126.9	331.4	315.1	444.5	trace
Linoleic	309.9	944.8	1866.5	2571.7	2044.1	20.7
Linolenic		54.4	39.0	110.7	462.7	1.8

Table 2. Changes in distribution of fatty acids during storage for one year at −17.8°C (acids calculated as weight of palmitic acid in mg in 1500 g of fresh weight).

Original fatty acids calculated to palmitic acid	Phospholipid			Neutral fat			Free fatty acid		
	Enzyme-inactivated	Untreated	Difference [a]	Enzyme-inactivated	Untreated	Difference [a]	Enzyme-inactivated	Untreated	Difference [a]
Palmitic	1230.5	603.0	− 627.5	641.3	880.4	+ 239.1	10.5	2563.4	+2552.9
Oleic	283.8	12.9	− 270.9	400.4	114.3	− 286.1	—	298.5	+ 298.5
Linoleic	2351.2	278.8	−2072.4	1868.8	863.8	−1005.0	18.9	1706.4	+1687.5
Linolenic	101.9		− 101.9	426.1	50.1	− 376.0	1.7	35.9	+ 34.2

[a] Difference is equal to the untreated minus the enzyme-inactivated.

FFA, converted to the equivalent weight of palmitic acid.

The data confirm earlier reports (Lee, 1954; Lee and Wagenknecht, 1951) by showing that a large quantity of acids in the FFA fraction of the lipids was obtained from the untreated (raw) material, when contrasted with the same fraction of the lipid material obtained from the sample treated to inactivate the enzymes before storage.

It appears that, as a result of changes taking place during storage, all of the several fatty acids in the phospholipid fraction showed a large loss in the raw samples as contrasted with the enzyme-inactivated samples. At the same time, the neutral fats in the same sample showed a loss for all of the unsaturated fatty acids in the raw when contrasted with those extracted from the enzyme-inactivated material.

Since the increase in FFA was large, it may be supposed that the larger amount of this acid came from the phospholipid fraction, while the lesser amount came from the neutral fats. The total disappearance of the major (in quantity) fatty acids (calculated as palmitic acid) from the phospholipid fraction of the raw material amounted to 3072.7 mg. The disappearance of these same acids from the neutral fat amounted to 1667.1 mg, again calculated as palmitic acid. This latter figure makes allowance for the 239.1-mg gain in palmitic acid, which was subtracted from the total quantity of the other major acids that disappeared from the neutral fat in the raw material during storage. This gives a net loss of 4739.8 mg of the major fatty acids from the phospholipids and the neutral fat of the raw material during storage.

It is possible that the increase in palmitic acid in the neutral-fat fraction of the untreated material during storage came from the unsaturated C_{18} acids from this same fraction.

Inspection of Table 2 shows that the fatty acids in the neutral fat and the FFA of the raw material show a net gain in total quantity of palmitic acid. It is possible that this increase came from the 3 unsaturated C_{18} acids. The oleic acid, therefore, which disappeared from the phospholipid of the raw material during storage, was added to that which disappeared from the neutral fat during the same period. From this sum, the amount found in the FFA was subtracted, and the result subtracted from the palmitic acid found in the FFA. This could be done because all the acids listed in Table 2 are calculated as palmitic acid. This same procedure was followed for the linoleic and linolenic acids. The sums of these latter were also subtracted from the palmitic acid, as was done with the oleic acid. The final result was a net gain in palmitic acid of 72.6 mg not accounted for by the palmitic acid originally present plus the conversion of the other fatty acids. It seems reasonable that this difference is within experimental error and that the fatty acids are formed by lipase action and perhaps beta oxidation (Rittenberg and Schoenheimer, 1937).

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Chemical Changes in the Juice Vesicles of Granulated Valencia Oranges

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(Manuscript received September 19, 1960)

SUMMARY

Granulation is a disorder of the Valencia orange in which the juice vesicles go through certain definite morphological and chemical changes. The larger the fruit, the more likely are granulated juice vesicles. The juice vesicles increase in volume with the subsequent hydration of the cell contents, followed by the gel formation of the pectic substances. The pectic substances and their respective methoxyl contents increased with the severity of granulation. The soluble carbohydrates and organic acids decreased as the mineral constituents and pH of the juice increased. The alcohol-insoluble fraction of the juice vesicles increased with the severity of granulation, and a highly significant correlation existed between the hardness of the juice vesicles and the alcohol-insoluble solids content.

The juice vesicles of the Valencia orange are subject to a physiological disorder known as granulation. In some years it has caused considerable economic loss to California growers and processors in that the abnormality affects unfavorably both the quality and quantity of the juice. The term "granulation" as used herein refers to a condition, usually, but not always, confined to the stem-end half of the fruit, in which at least a portion of the juice sacs have become progressively enlarged, hardened, and light gray in color. It should not be confused with "dry juice sac" ("blossom-end granulation"), in which the juice sacs have become soft and shrunken instead of hardened and enlarged. Whereas granulation is mostly confined to the stem-end of the fruit, dry juice sac is usually found in the opposite end or near the center of the fruit. Dry juice sac looks more like freeze injury than like granulation.

Data (Bartholomew *et al.*, 1934, 1935, 1936, 1941a) already published have shown that some physiological and structural changes occurred during the development of granulated juice vesicles. These studies showed that (a) the volume of a granulated juice sac may increase until it is 2–3 times that of a healthy juice sac; (b) a granulated juice sac may be 20 times as hard to crush as a healthy juice sac; (c) the walls of the cells in the granulated juice sac may increase to many times their usual thickness; (d) gas bubbles that form in granulating juice sacs look like crystals, and it was probably for this reason that the affected fruits were once said to be crystallized; (e) although not much juice can be reamed from the badly granulated portion of the fruit, the granulated juice sacs actually contain a little higher percentage of moisture than the healthy sacs; (f) an increase of at least 200% in dry matter was observed when the dry weight per individual juice vesicle was determined.

It is strongly believed that granulation is not caused by a fungus, virus, or bacterium, but is definitely related to the physiology of the tree and fruit during growth. Consequently, an investigation was made of the chemical differences between the normal and abnormal juice vesicles of granulated Valencia oranges. The fact that granulated juice vesicles have undergone tremendous increases in volume and cell-wall thickness indicates that energy has been expended in performing biochemical reactions, with resulting changes in chemical constituents.

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METHODS

Stages of granulation. During granulation the juice sacs go through many progressive physical and chemical changes. For descriptive and analytical purposes the visible physical changes, such as volume, size, color, and hardness are used in segregating the granulated juice vesicle into early, medium, and late stages (respectively A, B, and C). In the figures, the healthy, or normal, juice sac is designated as H. The vesicles begin to enlarge abnormally with the initiation of granulation, reach their maximum size in stage B, and gradually decrease in volume to stage C, accompanied by collapse and desiccation. Therefore, the general properties of the three stages of granulation are enlargement and hydration of the juice vesicles (stage A); hardening of the juice sacs and thickening of the cell walls, as well as enlargement of the vesicles (stage B); and juice-sac collapse as typified by a softening and progressive disintegration of the cell walls. The collapse begins at about stage C and continues as more of the internal cells disintegrate, forming an internal cavity in the juice vesicles. The extent of the disintegration made it impossible to obtain adequate samples for many of the analyses of this stage.

Chemical methods. All fruit samples were sized and graded before they were cut and sliced for estimation of fruit quality with respect to intensity of granulation.

The alcohol-insoluble solids values were determined by the methods described by Sinclair and Jolliffe (1960).

The pectic material was determined quantitatively as calcium pectate by the method of Carré and Haynes (1922) with slight modifications. The methoxyl groups were determined by the saponification procedure of Romeo (1933).

Total soluble solids were determined with an Abbé refractometer. Total acidity, expressed as citric acid, was determined by titrating an aliquot portion of the juice with a standard solution of NaOH, with phenolphthalein as an indicator. The pH values were determined with a Beckman glass-electrode pH meter.

The sugar determinations were made by the Hagedorn and Jensen (1923a, b) method as modified by Blish (1933, 1934). The strength of the reagents used by Blish was satisfactory for determining the reducing and total sugars as glucose when the values ranged from 3 mg to 10 mg in 10 ml of citrus juice. When necessary, the samples were diluted so that the values fell within this range. This method was used because comparative tests showed that it was more rapid than the best of the copper-reduction methods, and that it gave comparable results for the quantity of sugar in the sample. The glucose factor of the reagents was determined with a sample furnished by the National Bureau of Standards.

The sample to be ashed was evaporated to dryness and charred in an open crucible, transferred to an ignition boat with dilute HCl, and dried in an oven at 95°C. The material was then ashed and brought to constant weight, at 450°C, in a combustion tube through which a slow stream of oxygen was passed. Each weighed ash sample was dissolved in water containing HCl, and was stored until the analyses could be made. Each sample was filtered immediately before analysis, and any appreciable residue was reburned and dissolved in 3–4 drops of concentrated HCl and about 15 ml of water. The solution was then filtered, the two filtrates were combined, and the solution was made to 100 ml in a volumetric flask. Aliquot portions were then removed to be analyzed for calcium, magnesium, sodium, potassium, sulfate, and phosphate, according to the Official Methods of Analysis (A.O.A.C.).

RESULTS AND DISCUSSION

Fruit size in relation to granulation. The more rapid and luxuriant the growth of the fruit, the more severe is granulation, especially in the larger fruit sizes (Bartholomew *et al.*, 1941b). The cutting and examination of approximately 60,000 fruits over a 7-year period showed very clearly that the larger the fruit the more likely it is to develop granulation (Fig. 1). It appears that the

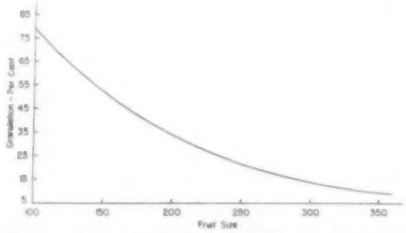

Fig. 1. Percentage of granulation, based on the number of fruits affected, in relation to fruit size (sizes are shown as number of fruit per standard wooden box).

large fruit become senescent sooner than the small fruit of the same bloom. Consequently, the juice vesicles of the large fruit have a tendency to initiate and develop granulation to a greater extent than those of small fruit. This is an experimental fact, but at present nothing is known as to what biochemical factors initiate this abnormal

development of the juice vesicles. Since juice sacs are individual metabolic units, it would be expected, and was found true, that juice vesicles adjacent to granulated ones are normal and healthy in both physical appearance and chemical composition.

The alcohol-insoluble solids of juice vesicles at different stages of granulation. This insoluble fraction of the juice vesicles, which includes the cell-wall constituents such as cellulose, pectic substances, hemicelluloses, proteins, and lignin, is designated as polysaccharides or acid-hydrolyzable materials. These substances, especially the pectic materials and hemicelluloses, appear to be closely related to one another. The amount of dry matter, on a per-vesicle basis, increases with the enlargement, hardening, and thickening of the cell walls of the juice vesicles during the granulation process. This condition of the vesicles led to the idea of determining the relation between the alcohol-insoluble fraction and the different stages of granulation. The results of this study (Fig. 2) show a significant increase in alcohol-insoluble materials during the progressive enlargement and hardening of the juice vesicles. Expressed on a dry-weight basis, the alcohol-insoluble fraction increased from 9.9% for the healthy to about 26.0% for the badly granulated juice vesicles (stage C).

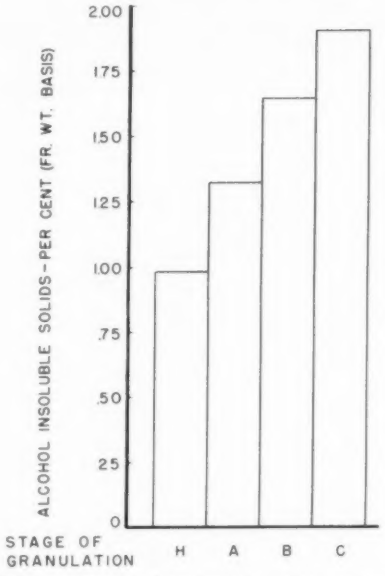

Fig. 2. The alcohol-insoluble solids of healthy and granulated juice vesicles at various stages of development. H, healthy; A, B, and C, stages of granulation.

Alcohol-insoluble solids in relation to juice vesicle tenderness. Kertesz (1935) introduced the method of measuring the alcohol-insoluble solids for determination of the maturity of green peas. Later, many investigators expanded this study to include the relationship between tenderometer readings and the alcohol-insoluble solids values (Lynch *et al.*, 1959). With respect to granulation of Valencia oranges, data have already been published on the crushing pressure required to flatten a juice vesicle to a 1-mm thickness at different stages of abnormal development (Bartholomew *et al.*, 1941). Since the alcohol-insoluble fraction increases with the progressive increase in the severity of granulation (Fig. 2) it was desirable to determine the relationship between similar crushing pressure values of the juice vesicles and their respective amounts of alcohol-insoluble materials (Fig. 3). The results showed a high correlation between hardness of juice vesicles and alcohol-insoluble solids content ($r = 0.953$; std. error $= 0.0789$). The alcohol-insoluble solids data are of in-

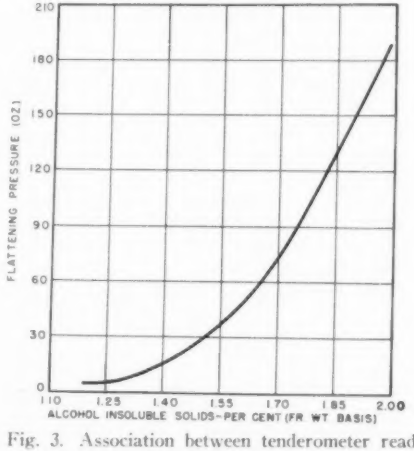

Fig. 3. Association between tenderometer readings (flattening pressure in oz) and the alcohol-insoluble solids of granulated juice vesicles.

terest not only because this characteristic of affected juice vesicles is associated with the increase in volume, cell-wall thickness, and vesicle hardness, but especially because it is indicative of the increased growth activity and senescence of the vesicles.

Total soluble solids and total soluble carbohydrates of granulated juice vesicles. The comparative amounts of total soluble solids, which are composed chiefly of soluble carbohydrates, were determined on healthy and granulated juice vesicles individually isolated from the stem-end fourth of the same fruits. When it is necessary to obtain samples of healthy juice sacs from ungranulated fruits, they must be taken from the stem-end fourth because the granulated sacs were taken from that portion of the fruit. This procedure takes care of individual compositional differences of fruits as well as the usual chemical gradient (Bartholomew and Sinclair, 1941; Hass and Klotz, 1935) of substances, principally sugars, that gradually increase in concentration toward the stylar end of the fruit. For comparative purposes, however, it is advisable to obtain samples of healthy juice sacs adjacent to or near the granulated vesicles from the same portion of the same affected fruits. The data in Fig. 4 show that the juice from the normal, or healthy, vesicles was noticeably higher in total soluble-solids content than that from granulated vesicles. Consequently, the concentration of total soluble sugars decreased as the severity of granulation increased. In all samples analyzed, the concentration of total sugars was higher in stage A than in stage B of granulation.

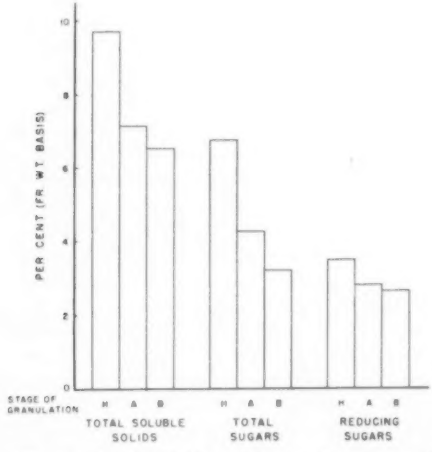

Fig. 4. Total soluble solids, total sugars and reducing sugars of granulated juice vesicles at two stages (A and B) of development.

Free acids and pH changes in granulated juice vesicles. The rates and types of reactions in juice vesicles are highly correlated with pH, which in turn is directly related to the free and combined acids in the juice. These factors, considered collectively as the buffer system of the fruit, are important in regulating, to some extent, the proper metabolic reaction in the juice vesicle. The relative amounts of free acids (titratable with standard NaOH) and active acidity (pH) in healthy and granulated juice sacs were determined from an aliquot of the same juice. The data in Fig. 5 show that titratable acidity decreased and pH values correspondingly increased with the advance of granulation. It should be mentioned that the pH value of orange juice bears a definite relation to titratable acidity, if compared over a wide range of acid concentration. This relation is not so definite, however, over shorter ranges of acid concentration; two samples having fairly larger differences in total acidity may have the same pH value, the condition depending on the kinds and amounts of buffer salts present.

The factors that control the pH of citrus juices are the same as those that control the pH of solutions of weak acids and their salts, and are not affected to any great extent by dilution. In such systems, the dissociation constant and the ratio of the acid to salt determine the approximate pH; and since this ratio is not changed by dilution, the change in pH is relatively small. This relationship is only approximate and sufficient for most practical purposes, but it is not entirely true. In addition to the ratio of acid to salt, the total concentration of ions in the system is involved in the pH changes of a buffer soluion on diluting with water. The activity coefficients change with the ionic strength. At ionic strengths greater than 0.1 normal, the difference between the individual ions in the systems becomes so great as to prohibit the use of an average

activity coefficient. Pure solutions of weak acids can undergo considerable dilution without any great change in pH provided the dissociation constant (Ka) is small and the undissociated residue sufficiently high to keep Ka constant.

Pectic substances and the methoxyl contents of healthy and granulated juice vesicles. Determinations were made on the comparative amounts of pectic substances in healthy and granulated juice vesicles. The healthy vesicles were isolated from the pulp near the stem end of ungranulated fruit, since obtaining sufficient sample for pectin analysis was difficult from the stem end of the granulated fruit and it is essential to use a single end because of gradient factors. The granulated juice sacs (stages A and B) were isolated separately and taken from near the stem end of granulated fruits. In each test the healthy and granulated sacs came from the same lot of fruits. The entire juice vesicle was used in each case. All segment-wall tissues were excluded. The results (Fig. 5) are calculated on a fresh-weight basis and represent the pectic substances, soluble and insoluble, determined as calcium pectate (Sinclair and Jolliffe, 1960). The data show about twice as much pectic material in granulated (stage B) as in healthy juice vesicles.

The degree of esterification of the carboxyl groups is shown by the amounts of

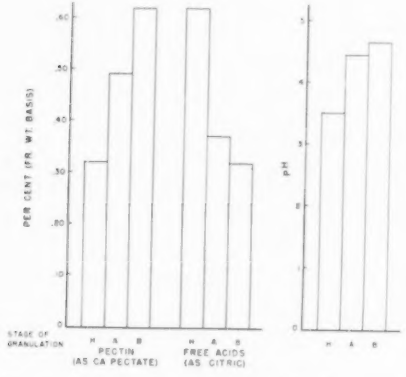

Fig. 5. Increase in calcium pectate, decrease in free acidity with corresponding increase in pH of granulated juice vesicles. H, healthy; A and B, stage in granulation.

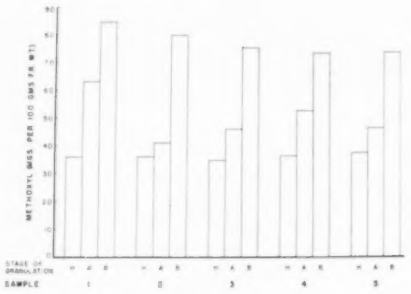

Fig. 6. Methoxyl content equivalent to 100 g of fresh weight of healthy and granulated juice vesicles (stages A and B).

methoxyl reported as mg per 100 g of fresh weight of juice vesicles (Fig. 6). It should be emphasized that the methoxyl values were actually determined on the alcohol-insoluble fraction of the healthy and granulated vesicles. The results show a progressive increase in methoxyl content with severity of granulation. The greatest variation between samples occurred in vesicles in stage A. The healthy or normal vesicles appeared to be slightly high in methoxyl content since they were obtained from affected fruits and located adjacent to or near granulated vesicles. The average of many determinations shows that healthy and granulated vesicles (stage B) had respective methoxyl contents of 3.15 and 4.25% of the alcohol-insoluble solids.

Mineral constituents of healthy and granulated juice vesicles. The principal inorganic constituents accumulated in abnormal amounts in the granulated juice vesicles. This fact is demonstrated for the five mineral constituents determined on the ash of samples taken from both healthy and affected vesicles (Fig. 7). The amounts of calcium and magnesium are especially large, there being an average of only 46.9% as much calcium and 59.1% as much magnesium in the healthy juice sacs as in the granulated ones. Furthermore, the granulated vesicles contained 43.7% of total calcium as water-insoluble calcium, whereas the average for healthy vesicles was 34.0%. Potassium was higher than any of the other inorganic constituents in the ash of both healthy and affected vesicles, but amounts of potassium were greater in the granulated tissues.

It must be noted that the mineral constituents are calculated as percentages of dry weight. As noted elsewhere in this paper, the concentration of soluble carbohydrates and organic acids decreases in the juice vesicles with an increase in severity of granulation, thus decreasing the dry weight and resulting in increasing the mineral content. It is not intended, however, to indicate that the increase in mineral constituents in the granulated vesicles is due entirely to the changes in dry matter.

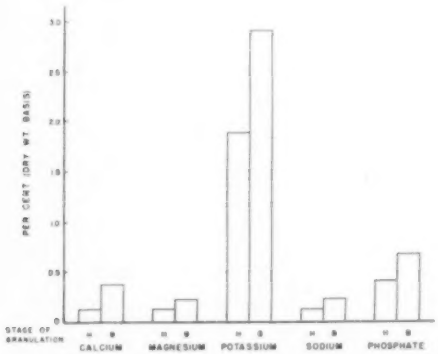

Fig. 7. Comparison of the mineral constituents of healthy and granulated juice vesicles (stage B).

Biochemical mechanism of granulation. Granulation begins by an increase in volume of the juice vesicles with hydration of the cell contents, followed by gel formation of the pectic substances. Gelation is a process through which all juice sacs pass before reaching the initial condition of stage C, the point where the juice vesicles begin to disintegrate and desiccate. The experimental evidence presented indicates that the gelation is the result of the combination of the divalent calcium ion with the pectic materials to form hydrated calcium pectate. As the concentration of sugars and organic acids decreases, the pH of the juice increases, with an increase in the mineral constituents available for salt formation. With diminution of these materials, the gelation process in the affected vesicles does not depend on the high sugar content or low pH, but probably depends on the presence of divalent cations (such as calcium) to react with any available amount of demethoxylated pectic materials.

As usually understood, gel formation requires four constituents: sugar, acid, pectin, and water in a definite relationship to each other. In this particular system, pectin is the gelling agent, the sugar and acid are the agents that bring about this physical transformation, and the water acts as a solvent for the other three constituents. In granulated juice vesicles, however, these four ingredients are not present in the right proportions for this particular type of gel. The concentrations of sugar and acids in the affected vesicles are insufficient, but enough pectic materials are present with free carboxyl groups to form gels in the presence of divalent cations. Both the pectic substances and their respective methoxyl contents increased with the severity of granulation. This increase in percentage of methylation of the pectic material would generally be associated with greater pectin solubility; however, sufficient cations are available to overcome this effect and cause gelation.

The alcohol-insoluble solids, which are chiefly cellulose, lignin, pectic substance, and hemicelluloses, double in amounts in the later state of granulation, as a result of the thickening of the cell walls and the increase in the structural elements of the affected vesicles. As a result of chemical action, gas bubbles (mostly carbon dioxide) form as the vesicle contents disintegrate and desiccate, leaving hollow spaces in the vesicles (Bartholomew *et al.*, 1941; Turrell and Bartholomew, 1943). It should be pointed out that this final stage of granulation is manifested by disintegration of the enlarged thick-wall and thin-wall cells, resulting in vesicles that have collapsed and become fibrous and tough (Bartholomew *et al.*, 1934, 1935, 1936).

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The Nutritive Value of Maine Sardines.[a] I. Chemical Composition

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(Manuscript received September 30, 1960)

SUMMARY

Maine sardines, processed by steaming or deep-fat frying, were evaluated in terms of a number of nutrients. The product is a good source of a number of nutrients, including protein, unsaturated fatty acids, fluorine, and calcium. Some indication was found of an exchange between the packing oil and sardine lipid. The sample processed by deep-fat frying had a slightly increased fat concentration and a concomitant decrease in water.

INTRODUCTION

Over the past five years, through an intensive research program conducted by the Research and Development Laboratories of the Maine Sardine Industry, the quality of sardines produced in Maine canneries has improved markedly. The quality control and grading program has been described by Clark (1959).

Because information is scant on the nutritive value of sardines produced in Maine, a comprehensive study was undertaken of the nutrient content of Maine Sardines, using modern analytical techniques.

MATERIALS AND METHODS

Samples: The test material was case lots of sardines selected randomly from the 1956 pack of five processors. All material was refrigerated at 38°F on arrival at these laboratories.

Sample preparation: After random selection from each case of a given processor, the cans were allowed to come to room temperature and opened, and the contents were drained for 10 min on a wire screen supported by a glass funnel. These drained sardines were considered to represent the product "as eaten." [b]

The drained fish were placed in a Waring Blendor and homogenized for 3 min in 500 ml of water. Aliquots were taken from this mixture for analysis.

Assay methods: Moisture was determined by the toluene distillation technique (Methods of Analysis, 1955 f).

The AOAC modification of the classical Kjeldahl nitrogen analysis was used to determine the protein concentration of the sample (Methods of Analysis, 1955 a).

Fat was determined as a "crude fat extract" with acetone followed by ethyl ether as the solvent system (Joslyn, 1950). Since the sardines were not completely free of adhering packing oils, this extract contained a small amount of these oils.

Ash was determined by combustion according to standard methods.

The loss in weight on combustion of the alkali and acid-insoluble materials isolated from defatted aliquots of the drained sardines represented the crude fiber content (Methods of Analysis, 1955 e).

Iron was measured colorimetrically by the Hahn (1945) method.

Chloride (Methods of Analysis, 1955 d) and calcium (Methods of Analysis, 1955 b) were determined titrimetrically, and phosphorus was measured as the pyrophosphate (Methods of Analysis, 1955 c).

Fluorine was estimated by the Fine and Wynne modification of the Willard-Winter technique (Fine and Wynne, 1959; Jacobs, 1958; Methods of Analysis, 1955 g).

Amino acids—methionine, tryptophane, threonine, lysine, valine, phenylalanine, leucine, and isoleucine—were determined microbiologically (Burton-Wright, 1952).

Because of the possibility of an exchange between the sardine oil and the packing liquids during processing and subsequent storage, the extracted sardine fats could not be said to represent pure sardine oil. To characterize the sardine oils

[a] Contribution Number 408 from the Department of Nutrition, Food Science and Technology. Sponsored by a generous grant from the Maine Sardine Council and the William Underwood Company.

[b] These samples were prepared in this way because the Maine Sardine Council had found that the consumer generally drained the oil and ate only the fish.

and their packing liquids, iodine number (Methods of Analysis, 1955 h), free fatty acids (Methods of Analysis, 1955 i), refractive index, and cholesterol (Brown *et al.*, 1954; Methods of Analysis, 1955 j), were determined on both the sardine oils and the drained oils.

Unsaturated fatty acids were determined with the alkali isomerization procedure of Herb and Riemenschneider (1952, 1953). Since the exact composition and structure of the fish-oil fatty acids and their spectral constants have not been determined, the data are reported as per cent diene, triene, tetraene, and pentaene.

The thiochrome procedure was used for determining thiamine (Hennessy, 1941).

Riboflavin was measured with a fluorometric technique (Connor and Straub, 1941).

Microbiological assays were used for both pyridoxine (Methods of Vitamin Assay, 1951 b) and niacin (Methods of Vitamin Assay, 1951 a).

RESULTS AND DISCUSSION

In common with most fish products, sardines are a good source of protein (Table 1), ranging from 21.5% to 24.1% (about the same as meat). In addition, the pattern of the constituent amino acids is good (Table 2). Lysine in particular is present in high concentrations. This is a characteristic of many fish proteins.

The iodine values of the oil extracted from the sardines and the packing oils were essentially the same (Table 4), an indication of the exchange between packing oil and the oil in the sardines.

Further evidence for the presence of packing oils in the fish-oil extract is shown in the analysis of the unsaturated fatty acids (Table 3). Raw and steamed herring oils

Table 1. Vitamin, mineral and proximate composition of drained canned sardines.

	Brand				
	A	B	C	D	
Proximate analysis					
Moisture (%)	62.2	63.4	63.4	62.2	58.3
	± 2.2	± 3.9	± 3.2	± 2.8	± 2.1
Protein (%)	23.5	21.5	23.3	22.1	24.1
	± 0.6	± 1.6	± 1.0	± 0.4	± 1.2
Crude fat	11.24	11.34	11.32	11.22	15.4
Extract (%)	± 2.10	± 1.92	± 3.30	± 1.85	± 1.9
Ash (%)	4.78	3.35	3.60	4.05	3.58
	± 0.32	± 0.49	± 0.35	± 0.71	± 0.29
Crude fiber	trace	trace	trace	trace	trace
Minerals					
Iron (mg/100 g)	4.12	2.67	2.84	2.79	2.55
	± 1.03	± 0.32	± 0.33	± 0.37	± 0.40
Chloride (%)	1.56	0.885	0.832	1.19	0.796
	± 0.18	± 0.329	± 0.155	± 0.31	± 0.215
Phosphorus (%)	0.876	0.866	0.889	0.942	1.028
(AS P_2O_5)	± 0.184	± 0.234	± 0.256	± 0.137	± 0.097
Calcium (%)	0.398	0.483	0.437	0.450	0.540
	± 0.078	± 0.050	± 0.091	± 0.062	± 0.144
Fluorine (mg/100 g)	—	—	4.34	—	4.65
Cholesterol (%)	0.146	0.135	0.161	0.127	0.138
	± 0.016	± 0.002	± 0.020	± 0.016	± 0.013
Vitamins (mg/100 g)					
Thiamine	0.0200	0.0200	0.0208	0.0320	0.0376
Riboflavin	0.119	0.141	0.147	0.129	0.145
	± 0.040	± 0.014	± 0.012	± 0.021	± 0.027
Pyridoxine	0.234	0.241	0.233	0.242	0.228
	± 0.019	± 0.019	± 0.027	± 0.033	± 0.034
Niacin	5.27	5.39	5.07	5.25	6.15
	± 0.52	± 0.70	± 0.27	± 0.12	± 0.28

± Standard deviation of the mean.

Table 2. Amino acid content [a] of drained canned sardines.

	Brand				
	A	B	C	D	E
Amino acid					
Methionine	3.12	3.26	3.27	2.91	3.17
Tryptophane	.89	1.01	0.99	0.93	0.96
Phenylalanine	3.89	3.73	3.68	3.70	3.84
Threonine	5.19	4.13	4.81	4.84	4.73
Valine	5.44	5.90	6.00	5.46	5.39
Lysine	9.36	8.88	9.37	8.50	8.97
Leucine	8.54	9.00	8.44	8.44	8.76
Isoleucine	4.93	5.11	4.96	4.70	4.81

[a] Expressed as per cent amino acid in drained sardine protein.

Table 3. Estimated fatty acid content [a] (%) of the crude fat extract of drained canned sardines.

	Diene	Triene	Tetraene	Pentaene
Raw herring oil (winter harbor)	2.05	2.80	2.56	21.16
Steamed herring oil (winter harbor)	4.83	5.41	5.08	17.10
Canned sardines (Brand D)	19.51	3.74	3.82	11.16
Fish oil [b]	3.02	4.21	5.13	20.30

[a] Calculated from the equations of S. F. Herb and R. W. Riemenschneider; Anal. Chem. 25: 953 (1953).
[b] Value from reference above.

contain mostly pentaenoic acids. These results agree substantially with the data of Herb and Riemenschneider (1953). The oil extracted from drained canned sardines, in contrast, contained a much higher proportion of dienoic acids and a relatively lower proportion of pentaenoic acids. It thus appears that a relatively large amount of the packing oil remained in the so-called "drained" sardines, and in actual fact is consumed with the sardine itself.

The packing oil may contribute also to the fat content of the drained product. These values ranged from 11.2% to 15.4%, with most of the samples containing about 11% fat (Table 1), about that of beef muscle meat. The fish oils, however, are more highly unsaturated.

Sardines are a good source of certain vitamins (Table 1). Thiamine concentrations ranged from 0.020 mg/100 g to 0.0376 mg/100 g. The concentration of riboflavin

Table 4. Characteristics of the crude fat extract of canned drained sardines and their packing liquids.

	Brand									
	A		B		C		D		E	
	Fish Oil	Pack Oil	Fish Oil	Pack Oil	Fish Oil	Pack Oil	Fish Oil	Pack Oil	Fish Oil	Pack Oil
Iodine number	125.3 ±5.7	122.8 ±2.9	131.6 ±4.4	122.4 ±3.9	126.8 ±0.8	126.3 ±3.3	133.3 ±2.4	127.6 ±3.9	129.1 ±2.5	125.9 ±3.5
Free fatty acids (% oleic)	4.60 ±1.55	0.28 ±0.09	5.32 ±1.04	0.13 ±0.01	4.31 ±0.60	0.16 ±0.06	5.30 ±2.05	0.13 ±0.04	2.73 ±0.54	0.14 ±0.08
Refractive index (25°C)		1.4728 ±0.0006		1.4727 ±0.0003		1.4730 ±0.0002		1.4734 ±0.0003		1.4725 ±0.0008
Cholesterol (%)	1.41 ±0.28	0.354 ±0.047	1.30 ±0.17	0.342 ±0.035	1.60 ±0.08	0.347 ±0.038	1.27 ±0.12	0.332 ±0.030	0.91 ±0.15	0.33 ±0.03

± Standard deviation of the mean.

varied from 0.119 to 0.147 mg/100 g, and that of niacin from 5.07 to 6.15 mg/100 g. If it is assumed that 100 g (1 can) of drained fish are consumed, these respective values represent about 10% and 25% of the daily allowance of riboflavin and niacin recommended for men (Natl. Acad. Sci. Natl. Research Council, 1953). Sardines are also a relatively good source of pyridoxine, ranging in concentration from 0.233 to 0.242 mg/100 g.

A significant part of the NRC allowance for men of iron and calcium can be supplied by 100 g of sardines (Table 1). Calcium ranged from 0.398 to 0.540 mg/100 g, about 50% of the allowance, whereas iron varied from 2.55 to 4.12 mg/100 g, about 30% of the daily allowance. Chloride was also present in significant concentrations; varying from 0.832% to 1.56%. Phosphorus ranged from 0.866% to 0.942%.

As anticipated, both of the samples analyzed for fluorine contained relatively large amounts, ranging from 4.34 mg/100 g for brand C to 4.65 mg/100 g for brand E. Seafoods are, of course, considered to be significant sources of fluorine.

The low crude-fiber content of these products is not unexpected (Table 1). As is true for most fish and meat products, the low fiber content implies a high degree of digestibility.

The concentration of the various nutrients was similar for all four samples studied, but a few differences were found—in each case significant, though small.

Sample A had a higher ash content than the other three brands, mostly in the form of increased iron and chloride. This might have been a function of the method used in preserving the fish before processing, or possibly due to the process itself. Greater salt concentrations in the preserving medium, for example, might tend to increase the chloride value.

The packing oil of Sample A had a higher content of free fatty acids. This hydrolysis of triglycerides of the oil may be a function of bacterial action or processing.

Sample E had reduced water and an increased fat concentration, probably a function of the process used in preparing the fish. Sample E was prepared by frying in oil before canning, as opposed to the steaming of the other four samples. This could have increased the oil concentration in these fish.

The nutritive properties of a food cannot be determined by physicochemical analytical techniques alone, it is true, but with this reservation, the results show that sardines, a relatively inexpensive foodstuff, are a potential source of many nutrients.

Acknowledgment

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Maple Sirup. XVI. Isolation and Identification of Compounds Contributing to the Flavor of Maple Sirup[a]

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(Manuscript received September 30, 1960)

SUMMARY

A major portion of the flavor was isolated from maple sirup by extraction with chloroform. From this was separated several pure crystalline compounds and several impure materials. Vanillin and syringaldehyde were definitely identified. These are compounds that could come from precursors in the decomposition of lignin, and this possible source of maple flavor is being investigated.

The distinctive flavor of maple sirup has kept this product selling at premium prices for many years. Many attempts have been made to imitate it with mixtures of vanillin, coumarin, and lovage, and extracts of coffee grounds, fenugreek seed, corn cobs, and hickory bark. Also, the reaction products of some reducing sugars and amino compounds have a maple-like flavor (Nelson, 1928). All, however, fall far short of true maple flavor, which has never been completely characterized. In 1925 Robinson separated a maple flavor fraction from maple sirup with chloroform, ether, or benzene. Skazin (1930) and Nelson (1928) separated vanillin-like products from ether and chloroform extracts. Risi and Labrie (1935) and Findlay and Snell (1935) suggested a relation of maple flavoring compounds to lignin. The most extensive attempt to identify maple flavor was made by Sair and Snell, in 1939. They separated a chloroform extract of a large amount of sirup into several gross fractions by the use of other organic solvents. These isolates, on further fractionation, yielded a number of compounds and mixtures of compounds. None, however, was identified definitely.

The main objective of research on maple products at this laboratory has been the development of modern processing methods for making high-quality maple sirup through improved control of the factors influencing flavor development. The earlier program included a study to determine the general chemical composition of maple sap and sirup. The current program contains a more intensive study of the chemical identity and origin of the flavor in maple sirup. This paper reports isolation of a flavor fraction from a good-quality maple sirup, and characterization of some of the compounds contributing to the maple flavor.

EXPERIMENTAL

Using information of Sair and Snell (1939) and Nelson (1928) on extraction of maple flavor, chloroform was used to obtain from maple sirup an extract, free of sugar and color, that contained the maple flavor.

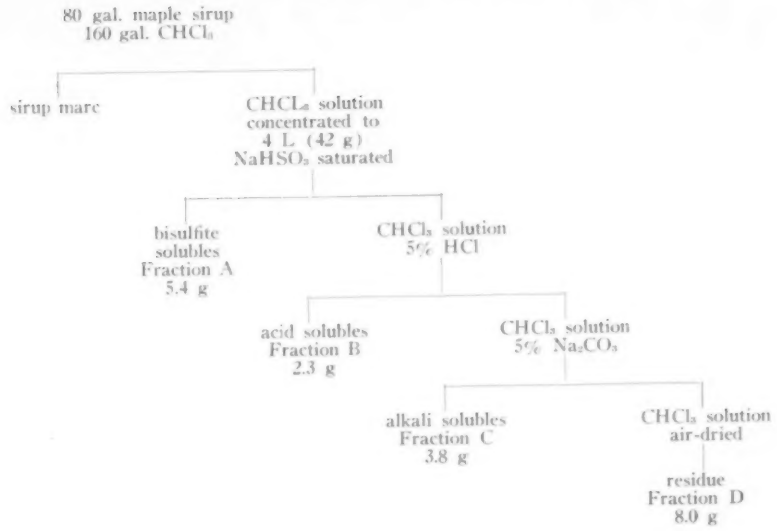
Procedure. Eighty gallons of maple sirup with distinctive maple flavor was divided into four 20-gal. portions. Each portion was extracted with two successive 20-gal. portions of chloroform. Extraction was accomplished by placing the sirup and chloroform in a 55-gal. drum and stirring for one hour. The stirred mixture was then let stand for several hours or overnight for the sirup and chloroform to separate into two layers, and then re-extracted with the second portion of chloroform. All of the chloroform portions were combined and concentrated at atmospheric pressure to a volume of 4 liters. Determination of the solids in an aliquot of the concentrated chloroform extract showed that 41.6 g of air-dried material had been obtained from the 80 gal. of sirup. The extracted sirup marc was stored for later work on color.

Fractionation of the Chloroform Extract. For a preliminary survey of the composition of the

[a] Presented at the 20th annual meeting of the Institute of Food Technologists, San Francisco, California, May 18, 1960.

[b] Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture.

Table 1. Flow sheet, chloroform extraction of maple sirup


80 gal. maple sirup
160 gal. $CHCl_3$
sirup marc
$CHCl_3$ solution concentrated to 4 L (42 g) $NaHSO_3$ saturated
bisulfite solubles Fraction A 5.4 g
$CHCl_3$ solution 5% HCl
acid solubles Fraction B 2.3 g
$CHCl_3$ solution 5% Na_2CO_3
alkali solubles Fraction C 3.8 g
$CHCl_3$ solution air-dried
residue Fraction D 8.0 g

chloroform extract a small aliquot was mixed with silicic acid, dried, and added to the top of a silicic acid column prepared from a slurry of Skellysolve B (mention of trade names does not imply endorsement by the Department over others not named). The column was eluted with a series of solvents giving increasing polarity from Skellysolve B, to chloroform, to ethyl alcohol. A large number of fractions were obtained. This procedure proved impractical because of the time required to obtain adequate amounts of separated materials. Therefore the following procedure was used to separate the original chloroform extract into four gross fractions for further purification in quantities large enough for identification tests.

Three 1000-ml portions of the concentrated chloroform extract were each concentrated on a steam bath to 500 ml. These were extracted by shaking in a separatory funnel with three 200-ml portions of ice-cold saturated sodium bisulfite solution. The extracted solution was held for later treatment. The combined bisulfite extracts were treated with solid sodium bicarbonate until frothing stopped and pH became 7. This decomposed the sulfite addition compounds removed from the original chloroform extract. The resulting solution was extracted with three 200-ml portions of chloroform, and these chloroform extracts were combined and evaporated to 250 ml. This chloroform solution of the sulfite addition compounds was then extracted with three 150-ml portions of ammonium hydroxide (1-10). The ammonium hydroxide extract was acidified with hydrochloric acid and re-extracted with chloroform to give a final purified solution of the compounds isolated through sulfite addition (Fraction A) containing 5.4 g of dry matter.

The sulfite-extracted chloroform extract was divided into three 500-ml portions. Each was extracted by shaking with three 250-ml portions of 5% hydrochloric acid. Again the extracted solution was held for further treatment. The combined hydrochloric acid extract was neutralized with sodium carbonate and extracted with chloroform. On air drying, this chloroform extract of the acid-soluble material yielded 2.32 g of a brown sirup (Fraction B).

The acid-extracted original chloroform extract was divided into 500-ml portions and each shaken with three 100-ml portions of 5% sodium carbonate and the water layers combined. This was neutralized with hydrochloric acid and re-extracted with chloroform. The resulting chloroform solution of the alkali-soluble compounds of the original chloroform extract was evaporated to 25 ml on a steam bath and allowed to go to dryness at room temperature, yielding a brown sirup (3.84 g) with a strong maple flavor. This was taken up in chloroform for storage (Fraction C).

The exhaustively extracted original chloroform extract was evaporated to dryness at room temperature and yielded a residue of 8 g (Fraction D). Substantial losses of material occurred dur-

ing fractionation, especially in the bisulfite treatment.

Compounds Isolated. a. The bisulfite addition compounds isolated in Fraction A were chromatographed on a column of silicic acid with a solvent of 70% Skellysolve B and 30% chloroform. Two fractions were obtained, both sweet in odor but definitely still impure. The first fraction was rechromatographed on silicic acid with a solvent of 95% Skellysolve B and 5% chloroform. The eluate from this column contained a compound that, when crystallized from chloroform–Skellysolve B, melted sharply at 80°C. The infrared curve for this compound was identical with that of synthetic vanillin. Its 2,4-dinitrophenylhydrazine derivative (DNP) melted at 271–272°C (recorded for the vanillin 2,4-DNP 270°C (Kremers, 1948)). The second fraction contained small faintly yellow crystals that, when purified by crystallization from Skellysolve B–chloroform, melted at 111°C (cor.). The 2,4-DNP derivative had the same melting point, 232°C, as that found for a known syringaldehyde 2,4-DNP. The infrared absorption curve obtained for the crystals was almost identical with that of syringaldehyde (Fig. 1).

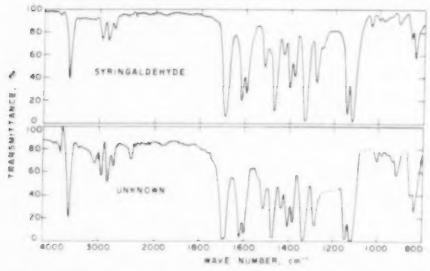


Fig. 1. Infrared absorption curves of syringaldehyde and flavor component.

b. The acid-soluble (Fraction B) part of the flavor isolate had a nut-like aroma. The material was chromatographed on a silicic acid column with a solvent of 37% chloroform and 67% Skellysolve B. The first, faintly yellow, band that was eluted had the intense nutty aroma of this fraction. The material was amorphous and will require further purification for identification. A second band eluted with the same solvent was highly fluorescent. It, too, lacked crystalline structure, and was devoid of aroma. A paper chromatogram showed that it was not closely related to the sugars. Its infrared absorption curve had many of the bands characteristic of vanillin and syringaldehyde found in the bisulfite fraction (Fraction A). Finally, a residual brown material was stripped from the column with ethanol.

c. The alkali-soluble fraction was the largest of the gross separations except for the residue left in the original chloroform extract after the treatment with bisulfite, acid, and alkali. Of all the fractions of the flavor isolate, only this portion had an intense aroma that closely resembled true maple. It could not be adequately separated into its pure components with the silicic acid chromatographic column used with Fractions A and B. Consequently, further study will be necessary for identification of its components.

d. Fraction D. The residue remaining in the original chloroform isolate after extraction with bisulfite, acid, and alkali was a gummy brown mass with a sweet taste. This will be analyzed further and reported in a subsequent paper.

Acknowledgment

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Dehydration in Model Systems: Cellulose and Calcium Pectinate

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SUMMARY

Cellulose, containing some resistant hemicelluloses, was prepared from the phloem of carrot tap-roots, and a similar extraction procedure was used for cotton fibers. A calcium pectinate gel was also made, and all three materials were dehydrated at two different rates and stored for 6 months. Proportionate crystallinity was determined on the basis of differences in moisture content, water vapor sorption, X-ray diffraction intensity, iodine adsorption, enzymatic digestibility, and density. In all cases, dehydration resulted in an increase in crystalline content of the samples, rapid dehydration seeming to produce somewhat greater crystallinity than slow dehydration. (Some spurious results with calcium pectinate are discussed.) Uniformly, all materials became more crystalline on standing, and the products of the two different drying rates attained virtually the same crystallinity values, per sample type, after 6 months. Calcium pectinate had the highest amorphous content, carrot cellulose the next highest, and cotton cellulose the lowest.

Textural quality in food products involves the properties of natural high polymers, such as cellulose, pectin, hemicelluloses, starch, etc. The physical chemistry of these high-polymeric systems is still a young, though rapidly expanding, discipline. As a consequence, there is little information on the manner in which polymers are changed during food processing. The effect of freezing or drying or even cooking on the behavior of macromolecules is still largely unknown.

Dehydration is one of the very ancient methods of food preservation. Because of the limited free-water content—and often high osmotic concentration—it is unusual for a desiccated product to sustain microbial growth. Moreover, dried food, being very light, is transported more readily than fresh food. There are many other advantages in dehydrating foods; there are also compelling problems. One of the significant problems with dehydrated commodities is a textural one: rarely will the product return to its original size and tenderness upon soaking in water.

An important reason for this lack of complete reconstitution, at least for fruits and vegetables, is the inability to make use of osmotic mechanisms to regain original turgor. This situation results from the death of the cell and the destruction of its organized cytoplasmic membranes during processing (Sterling, 1959; Thomas, 1935). However, because some volume increase does occur, it seems reasonable to expect that this increase is due to swelling of the hydrophilic materials in the cell. When proteins and starch are absent, these materials are almost exclusively in the cell wall.

The main constituents of parenchymatous cell walls are cellulose, hemicelluloses, and pectic substances (Bishop *et al.*, 1958; Jensen, 1960). The last presumably exists principally in the form of calcium pectinate (Bonner, 1936; Molisch, 1913; Personius and Sharp, 1938). All these substances are present as polymeric gels. To a varying extent, the gels will be composed of crystalline and amorphous domains, i.e., there will be regions of association and regions of non-association of the polysaccharide molecules. The swelling properties and chemical reactivities of the gels are due principally to their amorphous regions; the crystalline regions are usually unavailable for water uptake, and they are generally inaccessible to

chemicals (Hermans, 1949; Hessler and Power, 1954; Mark, 1940; Philipp *et al.*, 1947).

The present study has sought to determine the effect of dehydration, generally, on proportional crystallinity of polysaccharide gels of the cell wall. Specifically, it has involved comparison of rapid versus slow dehydration, and determination of the effect of storage after dehydration on the degree of crystallinity of cellulose and calcium pectinate.

MATERIALS AND METHODS

Materials. Two kinds of cellulose were used: cellulose extracted from carrot and cellulose of cotton fibers. Imperator carrots were washed and peeled. After both ends of the taproot were removed, the secondary phloem was separated from the other tissues and diced and ground. (No lignin is present in the cells of this phloem tissue.) The pulp was washed five times with ethanol on a Büchner funnel, and then extracted for 8 hours with ethanol-benzene (1:1) in a Soxhlet extractor to remove all fat-soluble materials. Afterward, the pulp was refluxed 2 hr with 2% HCl, washed 5 times with distilled water on a Büchner funnel, refluxed 2 hr with 2% NaOH, and then washed 10 times with hot distilled water. This treatment removed pectic substances, the less resistant hemicelluloses, and salts. The remaining pulp was assumed, however, to contain cellulose and some resistant hemicelluloses, thus being "cellulose" in the sense of Myers and Preston (1959). Cotton cellulose, in the form of absorbent cotton, was extracted in the same way.

A calcium pectinate gel was prepared from commercial "sodium polypectate" (Exchange Orange Products Co., Ontario, Calif.). The sodium pectinate was dispersed in distilled water (2 mg/100 ml) with a mechanical stirrer at 60°C. After complete dispersion, the sol was cooled to about 30°C. Next, calcium chloride was put in solution with water (10% w/v), and the solution was used in the ratio 0.27 g/1.00 g of sodium pectinate, to make the gel. Two paper towels were soaked in the $CaCl_2$ solution. One towel was then placed in the bottom of a glass dish with the free $CaCl_2$ solution. The sodium pectinate sol was poured slowly over the surface of the $CaCl_2$ solution and then covered with the second soaked towel. When the assembly had remained 36 hr at room temperature, a firm calcium pectinate gel had formed. It was cut into dice, about 7–10 mm on a side, and washed in several changes of distilled water for 24 hours at room temperature.

Methods of dehydration. The diced calcium pectinate gel was spread on trays, on which were also placed dice of fresh carrot phloem and hard cloth envelopes containing the wet pulp of carrot cellulose and the wet pulp of cotton cellulose. In the method of rapid dehydration, the samples were held about 10 hr at 185–190°F dry-bulb and 85–90°F dew-point temperatures (relative humidity of 0.5%), by which time the moisture content of the fresh carrot dice reached a value of approximately 4%.

Slow dehydration involved a dry-bulb temperature of 145–150°F and a dew-point temperature of 85–92°F (relative humidity of 9%) for 30 hours. At that point the moisture content of fresh carrot dice was about 10%. Then the dry-bulb temperature was lowered to 110°F, with a dew-point temperature of 50–55°F (humidity of 0.1%), for 10 hours, when the moisture content of the fresh carrot dice reached about 4%.

The samples were stored 6 months at 30°C in separate cans, under 15 in. of vacuum.

Analysis. The analytical techniques are designed to measure the relative proportion of amorphous and crystalline regions in the gel materials. Because they are largely unassociated, the molecules in amorphous regions will react more readily with water and will be more accessible to chemical reagents (Hermans, 1949; Mark, 1940; Philipp *et al.*, 1947). Except for 20 g of calcium pectinate dice, the samples were ground to pass an 80-mesh screen.

Moisture content. Ten g of powdered sample were weighed into tared aluminum dishes in duplicate and dried 10 hr at 70°C under 27 in. of vacuum. Results were expressed as percentage of the dry weight.

Swelling. Twenty g of calcium pectinate dice were put in a 250-ml volumetric flask. Distilled water was added to cover the dice, but not to fill the flask. After 24 hr at room temperature, the flask was filled to the mark with additional water. The flask was inverted and allowed to drain for 2 min into a graduated cylinder. The difference between the excess water and 250 ml was taken as the rehydrated volume of the sample.

Water vapor sorption. Sorption of water vapor was measured dynamically, with moisture-bearing nitrogen gas passing through the samples at 40 ml per minute. Oil-pumped gas was passed in series through 3 sulfuric acid-water solutions, two of which were in a 30°C bath. About 0.5 g of powdered sample was placed in duplicate in a container in the bath, so arranged that the gas stream passed through the samples in parallel. The sulfuric acid solutions were made up to give relative humidities (RH) of 70, 50, and 30%, successively used in descending order. (It was determined that the gas

stream had equilibrated to the proper moisture content in the first flask.)

After moisture equilibrium was reached (usually somewhat more than 24 hr), the sample and its container were weighed quickly. When the 3 series of weights had been obtained, the sample was dried 10 hr at 70°C under 27 in. vacuum, and weighed again. The water vapor sorbed was expressed in terms of per cent of the sample dry weight.

X-ray crystallinity. A weighed quantity of powdered sample was packed in duplicate in the central hole of a brass disk, and X-ray exposures were made according to the procedure outlined by Sterling (1960). From photometric traverses of the X-ray film, crystalline and amorphous indices were calculated by the method of Hermans and Weidinger (1948). Percentage crystallinity was determined on a nomogram constructed after the same authors (Hermans and Weidinger, 1949).

Iodine adsorption crystallinity. A sample of 300 mg was weighed in duplicate and treated according to the procedure of Hessler and Power (1954). Percentage crystallinity was calculated by the use of their equation.

Enzymatic digestibility. Commercial cellulase from *Aspergillus niger* (Nutritional Biochemicals Corp., Cleveland, Ohio) was dissolved in distilled water (4 g/150 ml) with 50 ml of a pH 4 citric acid–phosphate buffer. A suspension of 200 mg (dry basis) of cellulose sample was made with 5 ml distilled water and 10 ml buffer solution (pH 4) and allowed to stand 1 hr at room temperature. Then 5.0 ml of the cellulase solution and 1 ml of toluene were added to the suspension, which was shaken briefly and loosely covered with a rubber stopper. The suspension was placed in triplicate on a shaker at 37°C for 6- and 24-hour periods.

Each reaction mixture was then filtered, and the filtrate was hydrolyzed 2 hr with 4% HCl at 100°C. After neutralization with NaOH, the solution was diluted and the amount of glucose determined by the Bertrand (1906) method. Percentage digestibility is the ratio of the amount of cellulose hydrolyzed (glucose × 0.9) to the initial amount of cellulose.

Density. Since material in crystalline form can be expected to be denser than in less organized amorphous form (Hermans, 1949; Treloar, 1941), density measurements were made. About 600 mg of sample were taken and dried briefly (70°C under 27 in. of vacuum for 3 hours). This procedure removed all the free water. The dried sample was weighed into a pycnometer, and 20 ml of ethanol were added. After the pycnometer had stood 10 hr at room temperature, it was filled with ethanol, and the weight was taken at 20°C. Density was computed by the usual method.

RESULTS

A résumé of the whole series of experimental results is presented in Table 1. Although the moisture content is not uniform among the three different kinds of dried material, it is markedly constant for the celluloses, as between the two different dehydration methods, and it remains constant during storage. Thus, for comparisons made among the same materials, there is essentially no subsidiary effect due to a differing amount of moisture. It will be shown, in analysis of the other data, that differences in moisture content among the samples are related to differences in relative crystalline-amorphous composition. Thus, calcium pectinate appears to have the greatest amorphous content, carrot cellulose the next greatest, and cotton cellulose the least. Note that the slowly dehydrated calcium pectinate gel has a higher moisture content than the rapidly dehydrated one.

When the swelling volumes are measured, it is found, first of all, that the reconstituted volume of the calcium pectinate gels falls far short of the original volume. In both rapidly and slowly dehydrated gels, the swelling volume decreased during storage. Contrary to expectations, the rapidly dehydrated gel attains a somewhat greater volume than the slowly dehydrated one. No volumetric measurements were made on the cellulose pulp, which did not exist in large enough units to be handled in the manner of the dried calcium pectinate gel.

The water-vapor sorption data for the calcium pectinate gels followed the results with the swelling volume: higher moisture uptake in the rapidly dehydrated gel, and decrease in moisture uptake in both samples upon storage. As is generally true of sorption curves, more moisture is adsorbed at higher relative humidities than at lower ones. It should be noted that the calcium pectinate gel became visibly wet at 70% RH.

The effects of rapid and slow dehydration in the cellulose samples are the reverse of those in the pectinate gel. Initially, slowly dehydrated cellulose (both carrot and cotton) has a higher water uptake than rapidly dehydrated cellulose. However, this is much more marked in carrot than in cotton cellulose. In all cases, the amount of water-vapor sorption declines during storage. Most interesting is the fact that after 6 months, the values for rapidly dehydrated and slowly dehydrated cellulose are almost identical, even for carrot cellulose. Again, it should be noted that, as with the amount of moisture present after dehydration, in water vapor uptake the materials stand in the relationship: calcium pectinate > carrot cellulose > cotton cellulose.

Table 1. Analytical results for dehydrated model systems.

Sample	Dehydration rate	Storage time (months)	Moisture content	Rehydration volume (ml)	Water vapor sorption			Crystallinity (%)			Cellulose digestibility (%)		Density (g/ml)
					Relative humidity (%)			Sorption ratio	X-ray	Iodine adsorption	6 hr	24 hr	
					30	50	70						
Calcium pectinate	Rapid	0	6.70	48.0	20.4	38.2	70.1	....	26	96	....	....	1.83
		6	6.74	38.0	15.0	31.8	53.5	....	25	96	....	....	1.95
	Slow	0	8.00	40.8	18.2	30.5	63.3	....	24	100	....	....	1.83
		6	8.05	36.0	17.4	27.0	51.5	....	24	100	....	....	1.92
Carrot cellulose	Rapid	0	4.36	....	6.25	8.01	10.80	54	59	97	4.5	17.5	1.55
		6	4.39	....	5.62	7.43	9.46	58	63	98	3.0	11.0	1.54
	Slow	0	4.39	....	7.14	9.09	12.62	47	57	94	4.9	18.0	1.54
		6	4.40	....	5.48	7.32	9.35	59	62	99	2.8	11.2	1.58
Cotton cellulose	Rapid	0	3.07	....	3.53	4.99	7.00	71	70	96	....	3.0	1.52
		6	3.05	....	3.42	4.86	6.65	73	68	96	....	2.0	1.62
	Slow	0	3.10	....	3.60	5.06	7.18	71	68	93	....	4.0	1.53
		6	3.12	....	3.52	4.77	6.63	73	67	96	....	2.0	1.65

From the water-vapor sorption data, the sorption ratio (Hermans, 1949; Urquhart and Williams, 1925) of the celluloses may be computed. The sorption ratio is the ratio of the sorptive capacity of a sample to that of native cellulose at the same RH and on the same side of the hysteresis loop—in this case, during desorption. The sorption ratios for the three relative humidities were averaged for each sample, and crystallinity of cellulose calculated on the basis of an amorphous content of 30% for cotton cellulose. The values are presented in Table 1.

Crystallinity values, calculated from X-ray diffraction and iodine adsorption, are presented beside the sorption ratio crystallinities. For carrot cellulose, the value is lowest immediately after slow dehydration. (However, even that crystallinity value is greater than the X-ray crystallinity of air-dry fresh-carrot cellulose: 52%.) Again, after six months, the crystallinity values for slowly and rapidly dehydrated carrot cellulose are identical, and greater than they were initially. Differences due to dehydration rate and changes during storage are not as clear in cotton cellulose or in calcium pectinate. Note, however, that slowly dehydrated calcium pectinate has a higher crystallinity, by the iodine adsorption method, than rapidly dehydrated calcium pectinate. With respect to amorphous content, the samples stand in the series: calcium pectinate > carrot cellulose > cotton cellulose, according to X-ray data.

Because cotton cellulose experienced only negligible hydrolysis after 6 hours, only the results at 24 hours are presented for it (Table 1). It may be seen that slowly dehydrated cellulose is slightly more digestible than rapidly dehydrated cellulose and that digestibility declines during storage, reaching the same value for both rates of dehydration. Also, carrot cellulose is more digestible than cotton cellulose.

The density of the model systems is not significantly affected by the rate of dehydration. Except for rapidly dehydrated carrot cellulose, the density of all samples increased upon storage. A unique result here is the very high density of calcium pectinate, which is contrary to expectations based on the earlier data of the experiment.

DISCUSSION

In 1952, Baker and his co-workers attempted to analyze problems of reconstitution by investigating factors affecting the gross swelling property of a mixture of filter-paper cellulose, pectin, sugar, and acid. They varied the pH, the methoxyl content of the pectin, and other factors. However,

they were not concerned with the physical effects of the dehydration process or the change in the extent of swelling of each component of the cell wall.

On the basis of studying gel organization in the cell-wall polysaccharides, it is apparent that consistent relations have been documented here. It has been shown that calcium pectinate [even in the form of an ionotropic gel (Sterling, 1957a; Thiele and Andersen, 1955)] is more amorphous than carrot cellulose, which in turn is more amorphous than cotton cellulose. (The iodine adsorption results and the density determinations appear to run counter to this relationship. However, the iodine adsorption method gives results that are extraordinarily high—and may be invalid. The density measurements, being made in ethanol, cannot be interpreted completely with the concept of macroscopic density. Rather, as with water (Hermans, 1949), the hydrophilic ethanol molecules may fit in special places on the amorphous polysaccharide molecules, even perhaps with some compression, giving spuriously high readings, depending on the materials.)

Within the same type of material, however, changes due to storage are confirmed by virtually all the methods used here. All point to decreasing amorphous content and increasing crystalline content with time. Apparently, while the long-chain molecules lie close together, a slow "zipper" action takes place, and molecule-to-molecule bonding increases. After 6 months, it appears that the effects of different dehydration rates have been discounted—as though the materials tend to reach an equilibrium condition that is more or less specific for each.

A curious puzzle is the difference in the effects of rapid and slow dehydration on cellulose *vis-a-vis* calcium pectinate. Because dehydration is expected generally to increase crystallinity (Berkeley and Kerr, 1946; Kratky *et al.*, 1941) by bringing reactive polymer groups in closer proximity, it might be expected that rapid dehydration would yield a higher crystallinity than slow dehydration, particularly in the outer regions of the sample. This does occur in cellulose, in which slowly dehydrated material is more amorphous than rapidly dehydrated. However, except for the original moisture content and the X-ray data, the reverse relationship is indicated for calcium pectinate.

It is interesting to note that the determinations indicating that slowly dehydrated calcium pectinate is more crystalline than the rapidly dehydrated samples all involve the use of moisture. It should be recalled also that calcium pectinate actually becomes wet during sorption at RH 70%. A possible explanation may lie in the finding that calcium pectate takes up moisture *intramicellarly* as well as intermicellarly (Bettelheim, 1956). [Note that moisture uptake in cellulose reportedly occurs only in intermicellar, i.e., amorphous areas (Hermans, 1949; Mark, 1940).] Although the amount taken up by micelles is very likely much smaller than that by non-micellar material, it may be enough to disturb the intrinsic relationships. Because of its significance, this question should be investigated further.

The rehydrated volume of the calcium pectinate gel is considerably smaller than the original volume. Quite apparently the now closely adjoining molecules have established (hydrogen) bonds among themselves, and these bonds will not permit re-establishment of the original volume. The relative volume of calcium pectinate gel in the fresh cell wall is not known. Within the processed cell wall, it can be expected that after rehydration the relative gel volume for calcium pectinate will be similar to that of the rehydrated gel here. Since 20 g of dried, virtually nonporous, gel of approximate actual (i.e., macroscopic) specific gravity of 1.5 can rehydrate to a volume of about 38 ml, it is taking up about 25 g of water, or more than its own weight (if no air spaces are present). This uptake will have a significant effect in the swelling of the cell wall and the consequent reconstitution of dried tissue. The effect of cellulose (and resistant hemicelluloses) will probably be less, but it will also be important. It is incidentally noteworthy that the values for crystallinity of carrot phloem cellulose are similar to those for the cellulose of young asparagus fibers (Sterling, 1957b).

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Biochemistry of Pork Muscle Structure. I. Rate of Anaerobic Glycolysis and Temperature Change versus the Apparent Structure of Muscle Tissue[a]

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SUMMARY

Continuous recordings of pH and temperature changes, during post-mortem chilling, were made on 20 Danish Landrace carcasses. The continuous recordings from these carcasses depicted at least four distinct types of post-mortem pH patterns: 1) a slow gradual decrease to an ultimate pH of 5.7–6.3; 2) a gradual decrease to about 5.7 at 8 hr, with an ultimate pH of 5.3–5.7; 3) a relatively rapid decrease to about 5.5 at 3 hr, with an ultimate pH of 5.3–5.6; 4) a sharp, significant decrease to a pH of about 5.1 at 1½ hr, and a subsequent elevation to 5.3–5.6. The first 3 types were acceptable in structure, color, and water retention, whereas type 4 represented pale, exudative tissue with soft, inferior structure. The violent nature of the post-mortem changes in tissue with the type 4 pH pattern were discussed, as well as the possibility of a more rapid onset of rigor mortis in this tissue. Although the pH values of the pale, exudative tissue were elevated during chilling, it appeared that they remained generally lower, ultimately, than the normal tissue. On the basis of comparisons between muscles, it is suggested that in addition to chemical composition, the chilling rate of the individual muscle may also be an important factor in determining pH pattern and ultimate muscle structure.

Chemical changes in muscle tissue after death include the disappearance of creatine phosphate, resynthesis and disappearance of adenosine triphosphate (ATP), and production of lactic acid (Bate-Smith, 1948; Bate-Smith and Bendall, 1956; Bendall, 1951; Lawrie, 1953; Marsh, 1954). These changes are normally accompanied by a parallel drop in pH and a gradual reduction in the water-binding properties of the muscle tissue (Bate-Smith, 1948; Callow, 1938; Hamm, 1955, 1959; Lawrie, 1953; Marsh, 1954; Wismer-Pedersen, 1959a). Briskey *et al.* (1959) reported no significant differences in the pH of the musculature at the time of bleeding, though 40 minutes later the pH values were significantly ($P<0.05$) lower in muscles that were ultimately (24 hr) low in pH, pale in color, watery in appearance, and soft in structure. The pH of pork muscle after cessation of post-mortem glycolysis (ultimate pH) (Callow, 1938), usually ranges from 5.3 to 6.3. Lawrie *et al.* (1958), however, reported the occurrence of pale, exudative tissue with an abnormally low pH value (4.78–5.10) and suggested that this condition might be related to the extent and rapidity of the breakdown of ATP. Wismer-Pedersen (1959) likewise implied that these peculiarities arise from differences in the rate of pH decrease immediately post-mortem. A rapid pH decrease, seemingly due to an accelerated glycolytic rate, created low pH values (pH_1) at high muscle temperatures. It was further suggested by Briskey (1959a) that the lowest pH achieved by the tissue had a greater influence than its ultimate pH in producing soft, pale, exudative tissue. More recently, Wismer-Pedersen and Briskey (1961) reported continuous patterns of pH depressions and

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elevations, and intimated that these phenomena initiate a change in configuration of the muscle proteins—with a resultant decrease in color intensity and water-holding capacity.

This paper shows the influences of glycolytic velocity on certain pork muscle characteristics.

EXPERIMENTAL

Experiment I. Determination of pH patterns and simultaneous changes in glycolytic components. Twenty Danish Landrace pigs ranging in weight from 85 to 95 kg were used. The pigs were transported to the Roskilde Bacon Factory, where they were electrically stunned and slaughtered.

Physical determinations. A Radiometer pH meter (Model 24) was used to determine pH values at 15-min intervals to the 1-hr period. Subsequently the carcasses were transferred to the Danish Meat Research Institute and held at 3°C and 90% humidity for continuous recordings of pH and temperature for 1- to 24-hr periods. These continuous and simultaneous recordings were facilitated with a specialized pH meter (Radiometer, model no. 31; electrode-elector type ELS 31; electronic recorder type 153, Honeywell). Two pairs of pH electrodes (glass electrodes G 2326; calomel electrodes 4116) and thermocouples were uniformly placed in the medium of the *longissimus dorsi* of the left side at the area of the second lumbar vertebra and permitted to remain in position for 24 hr with regular adjustment. Careful tests have not revealed any indications of artifacts arising from this procedure. Samples of the *longissimus dorsi* from the 12th rib to the last lumbar vertebra, of the right side, were secured for analytical analyses, at 15-min intervals to 2 hr; 2-hr intervals to 12 hr; and 6-hr intervals to 24 hr. Sections ¼ in. thick from the exposed muscle areas were discarded before the individual collection periods.

Loose-water numbers were determined by the procedure of Wismer-Petersen (1959a). The *longissimus dorsi* muscles were ultimately classified in accordance with the muscle classification system described by Briskey *et al.* (1959).

Chemical determinations. The samples for chemical analyses were secured at the above-mentioned intervals and immediately frozen in dry ice. The frozen samples were subsequently analyzed for total glycogen by the procedure of Brand (1936); trichloroacetic-acid-soluble glycogen as outlined by Meyer and Hershberger (1957); lactic acid essentially as reported by Barker and Summerson (1941); and labile-P (7'P) as suggested by Bate-Smith and Bendall (1947). Fresh tissue samples were also secured and analyzed for preformed ammonia by a slight modification of the method of Convey (1939).

Experiment II. Comparisons and implications of pH and temperature patterns in five pork muscles of various anatomical positions. These studies were conducted simultaneously with experiment I and used the same carcasses and sample material. Six pairs of pH electrodes and thermocouples, as described in experiment I, were uniformly placed in the medium of the following muscles of the left side at the 1-hr period (Briskey *et al.*, 1958).

A) *longissimus dorsi*, 7th thoracic vertebra; B) *serratus ventralis;* C) *pectoralis profundus;* D) *longissimus dorsi*, 2nd lumbar vertebra; E) *semimembranosus;* F) *biceps femoris* (Fig. 1). The electrodes and thermocouples were permitted to remain in position for 24 hr for continuous recordings of pH and temperature. Samples from each of these muscles (right side) were secured (intervals, experiment I) for determination of loose-water, nitrogen, ether-extract, moisture, and lactic acid as previously described (Wismer-Pedersen, 1959a).

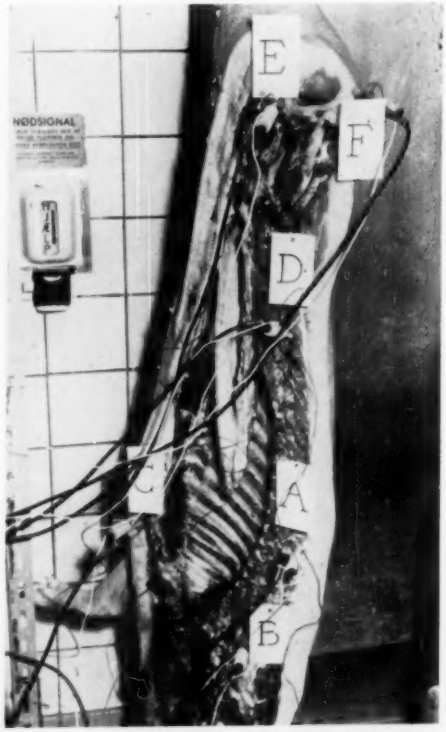


Fig. 1. Electrode and thermocouple insertion areas.

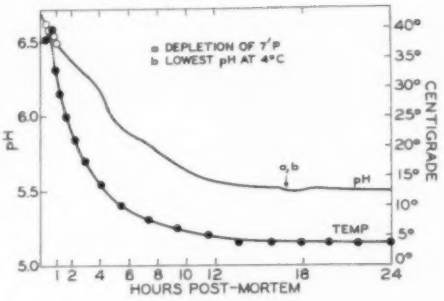
RESULTS

Experiment I. Determination of pH patterns and simultaneous changes in glycolytic components. The continuous recordings from these carcasses revealed at least four distinct types of post-mortem pH patterns: 1) A slow gradual decrease to an ultimate pH of 5.7–6.3. 2) A gradual decrease to approximately 5.7 at 8 hr, with an ultimate pH of 5.3–5.7. 3) A relatively rapid decrease to about 5.5 at 3 hr, with an ultimate pH of 5.3–5.6. 4) A sharp, significant decrease to a pH of about 5.1–5.4 at 1½ hr, and a subsequent elevation to 5.3–5.6.

The pH patterns and concomitant changes in certain glycolytic components in the *longissimus dorsi* for types 2, 3, and 4, above, are respectively in Figs. 2, 3, and 4. Fig. 2 depicts the normal features of a slow rate of anaerobic glycolysis (type 2 muscle). The glycogen contents were extremely variable, but a majority of the type 2 samples contained a relatively low content of total glycogen. The glycogen conversions, although rapid within the 1st hr, showed gradual change to the 18-hr period. Similarly, the lowest pH values were not attained, and marked depletions of labile-P were not apparent, until the temperature of the muscles approximated 4°C. Considering that only moderate extraction procedures were used, it is noteworthy that a relatively high percentage of the glycogen was readily soluble in cold 10% tricholoracetic acid. The lactic acid concentrations increased gradually to the 12-hr period, accompanied by relatively similar increases in loose-water and ammonia.

The liberation of ammonia from this type of glycolysis was seemingly complete at about the 10-hr period. Muscle heat was removed at a rate that gave a decrease in temperature of 8°C per hr during the initial 2-hr period. These muscles were ultimately firm in structure (classification 3—normal), high in water-binding capacity (LW 460), and average in color intensity (17–21, Elrepho Reflectance Units) (Wismer-Pedersen, 1960).

Fig. 3 shows the average features of a relatively rapid rate of anaerobic glycolysis (type 3 muscle). Although the glycogen concentration varied within a rather narrow range, it was rapidly converted to other glycolytic products during the 1st 15-min period. The lactic acid and loose-water concentrations were markedly and significantly increased during the initial 2-hr period when compared to the type 2 muscle (Fig. 2). The liberation of ammonia in this type of muscle, however, showed a close resemblance to the type 2 muscle. It is of further interest to point out that the muscle attained its lowest pH as the temperature approximated 25°C. Muscle heat was removed at a rate that gave a decrease in temperature of 5°C per hr during the initial 2-hr period. These muscles were



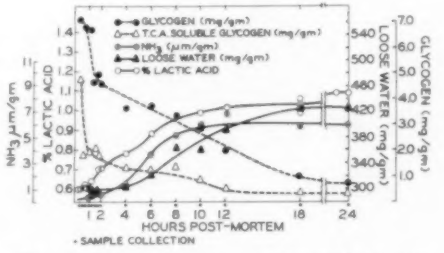
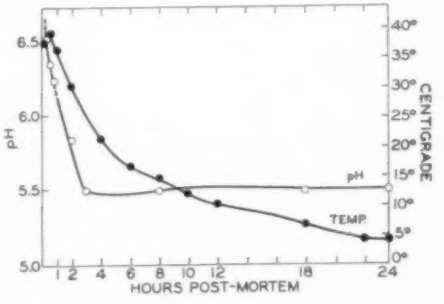


Fig. 2. Type 2 muscle. Characteristic post-mortem changes in pH, temperature, and glycolytic components.



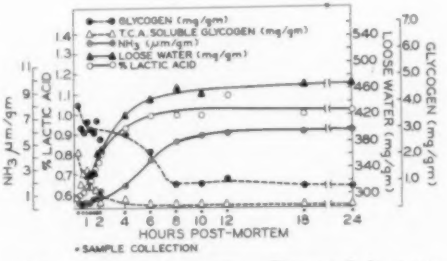


Fig. 3. Type 3 muscle. Characteristic post-mortem changes in pH, temperature, and glycolytic components.

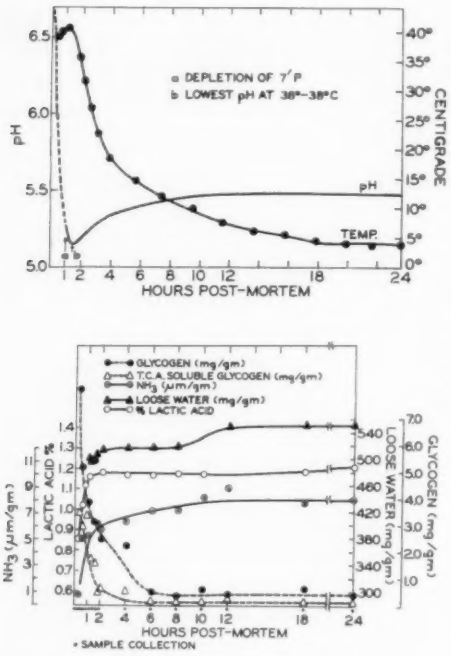


Fig. 4. Type 4 muscle. Characteristic post-mortem changes in pH, temperature, and glycolytic components.

moderately firm in structure (classification 3—normal), acceptable in water-binding capacity (LW 460–470), and average in intensity of color (17–22, Elrepho Reflectance Units).

The data in Fig. 4, however, characterize the violent nature of the anaerobic glycolysis in the type 4 muscle. Although the glycogen concentrations were unusually high, only a limited percentage of the glycogen was readily soluble in cold 10% tricholoroacetic acid. The pH decreased to 5.1–5.2, while the muscle temperature remained at 36–40°C. A rapid depletion of labile-P also occurred as the muscle attained its lowest pH. This action simultaneously initiated the immediate and complete production of lactic acid and a sharp increase in the quantity of loose-water. Similarly, the liberation of ammonia was especially rapid and approached completion within the initial 2-hr period. Muscle heat was removed at a rate that gave a decrease in temperature of 3.5°C per hr during the initial 2-hr period. These muscles were ultimately (24 hr) soft in structure (classification I—soft), "watery" in appearance (LW 480–520), and abnormally pale in color intensity (24–30, Elrepho Reflectance Units).

The dramatic changes in pH between these muscle types are implied in Fig. 5. This figure specifically demonstrates the relationship between pH, temperature, and time in the intact muscle. The observations on each line represent values at the points of collection over an 18-hr period. At the 4th collection period (1 hr post-mortem) the pH of the type 4 muscle had decreased to 5.2, while muscle temperature remained at 37°C. Conversely, the type 3 muscle showed a pH value of 5.9 and had decreased in temperature to 33°C. The type 2 muscle, however, retained a pH value of 6.4 but had decreased in temperature to 29°C. As would be expected, the muscles showing the greatest evolution of heat (Fig. 6) also reflected the most rapid decrease in pH. Subsequent elevations in pH seemed to occur simultaneously with the sharp decrease in temperature.

Fig. 6 illustrates the average temperature curves (logarithmic scale) for these carcasses. These data

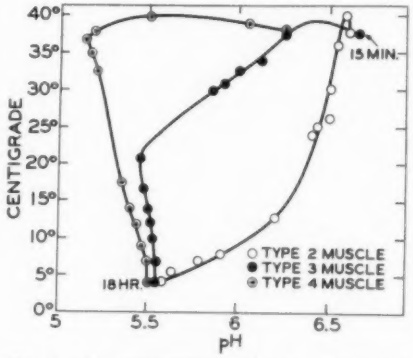


Fig. 5. pH, temperature, and time relationships. Consecutive observations for each muscle type were made at similar post-mortem periods.

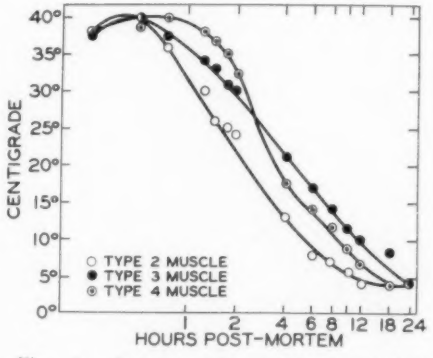


Fig. 6. Average post-mortem temperature. Curves, logarithmic scale.

imply that the greatest change in muscle temperature, as a result of glycolytic rate, takes place between 30 and 90 minutes. Subsequently, the temperature in type 4 tissue decreases rapidly and drops below the temperature of the type 3 tissue at the 3-hr period.

Experiment II. Comparisons and implications of pH and temperature patterns in five pork muscles of various anatomical positions. Muscles from individual carcasses varied significantly (Table 1) in the incidence of the four pH patterns previously described. Incidence of the type 4 pH reduction (rapid decrease resulting in pale, exudative musculature) was highest in the *longissimus dorsi* and *biceps femoris*. Conversely, incidence of the type 1 pH pattern (slow decrease resulting in normal to dark musculature) was highest in the *pectoralis profundus* and *serratus ventralis*. Fewer differences were noted in the occurrence of the type 2 and type 3 pH patterns.

Table 1. Frequency of occurrence of pH patterns in five different pork muscles.

Muscle	pH pattern			
	Type 1	Type 2	Type 3	Type 4
Longissimus dorsi	5[a]	20	25	50
Pectoralis profundus	50	10	30	10
Serratus ventralis	30	10	40	20
Semimembranosus	20	10	30	40
Biceps femoris	10	20	20	50

[a] Expressed as a percentage of samples observed.

Analytical data on the nitrogen and fat contents of the various muscles are shown in Table 2. The *serratus ventralis* contained a significantly greater fat concentration and lower nitrogen percentage than the other four muscles.

Representative chilling rates for these muscles are depicted in Fig. 7. Although the chilling rates for all muscles varied with the rapidity of pH reduction, they were reasonably uniform except for the *pectoralis profundus*, which decreased in temperature at an accelerated rate. The loose-water and lactic acid concentration data from the five muscles of each carcass were plotted on the basis of pH pattern (Figs. 8, 9, 10). Except for the *serratus ventralis* of the type 2 pH pattern (Fig. 8) the relationships of these characteristics in type 2 and type 3 muscles were remarkably similar. Conversely, the data from the type 4 muscles (Fig. 10) showed a sharper angle—except for the *semimembranosus*. It was apparent that the *longissimus dorsi* and *biceps femoris* were most susceptible to increases in loose-water, as a result of greater increases in lactic acid.

Table 2. Comparison of the proximate composition of five pork muscles.

Muscle	Nitrogen (%)	Fat (%)
Longissimus dorsi	3.56	2.30
Pectoralis profundus	3.41	1.40
Serratus ventralis	3.11*	6.30*
Semimembranosus	3.51	1.00
Biceps femoris	3.39	2.80

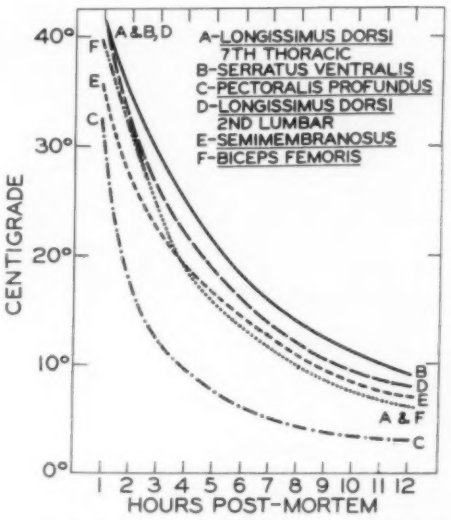


Fig. 7. Representative chilling rates for various muscles.

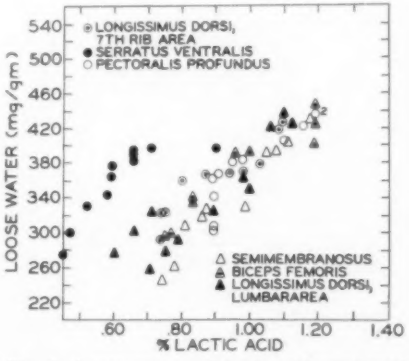


Fig. 8. Loose-water and lactic acid relationships in various muscles with type 2 pH patterns.

DISCUSSION

Determination of pH patterns. It should be emphasized that pH values were determined with probe electrodes (Wismer-Pedersen, 1959a). At each sample period, the pH values were also determined by macerating 1–2 g of muscle tissue in 10 ml of 0.005 *M* iodoacetate. When these values were plotted on the continuous pH curves there were similarities in direction, though there were slight differences in magnitude. The values determined with probe electrodes reflected not only greater depressions but also more marked elevations in pH at the 1–1½-hr period for the type 4 muscles when compared to samples macerated in iodoacetate. There appear to be several factors that should be considered in explanation of this difference, such as muscle condition (massaging of the cell), temperature, and, as previously suggested (Wismer-Pedersen and Briskey, 1961), state of rigor mortis. If the onset of rigor mortis were initiated while muscle temperatures remain high (above 90° F.), the fiber shortening, if any (Bate-Smith and Bendall, 1949), might have been more marked (6.25). Experiments were conducted to insert electrodes at various stages of the onset of rigor mortis. The results of these trials eliminate possible error from fiber shortening. Likewise, detailed studies disclosed no evidence of drift or unusual ionic dissociation at these temperatures. Since these depressions and elevations have also been reported (Briskey 1959b) with a Beckman model G pH meter, with individual sampling, it is assumed that, though these values may not be identical with those of macerated tissue, they can be used to reflect the relative pH value differences between these muscles for comparative purposes.

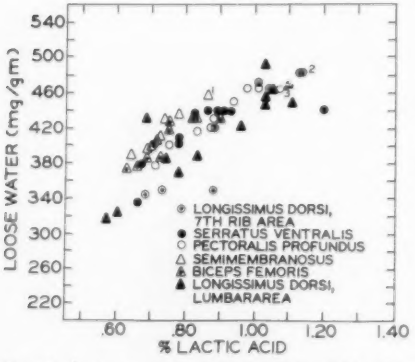


Fig. 9. Loose-water and lactic acid relationships in various muscles with type 3 pH patterns.

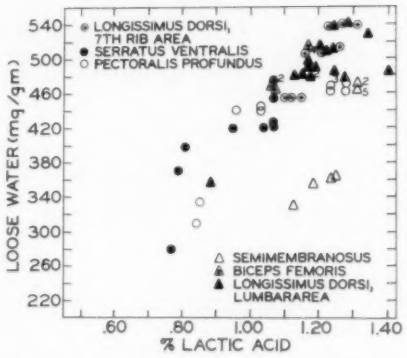


Fig. 10. Loose-water and lactic acid relationships in various muscles with type 4 pH patterns.

Chemical changes associated with pH patterns. The pH patterns reported associate pH elevation with inferior pork muscle structure. There are obviously samples, however, with inferior structure that do not reflect elevations in pH, or, in any event, whether they are elevated or not, retain relatively low ultimate pH values (Lawrie *et al.*, 1958; Ludvigsen, 1954; Briskey *et al.*, 1959). Likewise, in this experiment the samples with inferior structure, although elevated in pH, retained relatively low ultimate pH values.

Data for the type 2 pH pattern (Fig. 3) are in agreement with those reported by other workers (Bate-Smith, 1948; Briskey *et al.*, 1959; Callow, 1938; Howard and Lawrie, 1956; Lawrie, 1953). The disappearance of labile-P, once started, proceeded at a steady rate until about 30% remained, which is similar to the pattern shown in rabbit muscle (Bendall, 1951). This is in accord with work reported for the onset of rigor mortis in other species (Bate-Smith and Bendall, 1956; Lawrie, 1953; Marsh, 1952; Fremery and Pool, 1960). Similarly there was a gradual increase in lactic acid concentration with a concomitant increase in

loose-water. This relationship between lactic acid and loose-water, in domestic species, has been pointed out by several workers (Bate-Smith, 1948; Callow, 1938; Wismer-Pedersen, 1959a; Wismer-Pedersen and Briskey, 1961; Ingram and Ingram, 1955). Comparable relationships were also reported for whale muscle, Marsh (1952). As lactic acid was formed it was accompanied by a sizable decrease in fluid retention and a relatively rapid transition to a state of wetness. Hamm (1959) suggested that under normal conditions of anaerobic glycolysis, only about one-third of the decrease in hydration of beef muscle can be explained by the drop in pH. The implications and limitations of the acid-soluble glycogen data, as presented, are pointed out in a concurrent report (Briskey and Wismer-Pedersen, 1961). It should be emphasized that these data represent a single rapid extraction. More exhaustive procedures extracted greater quantities of glycogen. It is apparent, however, that there were at least differences in the availability of the glycogen for extraction. The factors influencing this availability as well as their implications are thoroughly discussed in the aforementioned report. The data in Fig. 4 show the extremely violent nature of this type of glycolysis. Although the total calories evolved during chilling might be comparable, regardless of pH pattern, it is obvious that in tissue with the type 4 pH pattern there is a sudden and drastic release of heat. When viewed from the standpoint of the extreme evolution of heat, retention of muscle temperature, and development of an especially low pH value at a time when the ATP is also nearly depleted, it becomes apparent that this is, undoubtedly, the point of structural change. This sequence of reactions appears to support the previous suggestion that rapid ATP depletion is associated with pale, exudative pork muscle tissue (Lawrie *et al.*, 1958). These points and other related factors will be discussed elsewhere in an explanation of the pH elevation. As previously reported (Wismer-Pedersen, 1959b), it appears that these factors contribute to configurational changes in the muscle proteins. It is recognized, however, that there may conceivably be inherent pathological differences as well as physiological and biochemical factors that contribute to the development of these structural alterations.

Comparison of pH patterns and associated changes in various muscles. The data on the incidence of various pH patterns in the five muscles (Table 1), which differ anatomically and functionally, are in agreement with the previous report (Briskey *et al.*, 1960) on the biochemical differences in these muscles. Additionally, the loose-water and lactic acid concentrations showed direct relationships but varied with type of pH pattern (Figs. 8, 9, 10).

The *biceps femoris* and *longissimus dorsi* were reported (Briskey *et al.*, 1960) to be remarkably similar in ultimate appearance and structure. These observations were similarly recorded in this experiment, and it was especially noteworthy that these two muscles also showed the highest incidence of the type 4 pH pattern and the lowest incidence of the type 1. Likewise (Briskey *et al.*, 1960), these two muscles possessed higher initial glycogen concentration, lower myoglobin concentration, and generally a lower fat percentage than the other three muscles.

Conversely, the *pectoralis profundus* and *serratus ventralis* contained greater myoglobin and fat quantities (Briskey *et al.*, 1960). Statistical analysis of the fat and nitrogen data in this experiment (Table 2) showed only the *serratus ventralis* to contain a greater fat concentration than the other muscles. Further support for implicating chemical differences in the incidence of these conditions can be found in the unpublished experiments of Wismer-Pedersen (1960). Both the *pectoralis profundus* and *serratus ventralis* were found to contain greater hydroxyproline contents than the other three pork muscles (Wierbicki and Deatherage, 1954). It therefore appears, that the lower incidence of the type 4 pH pattern in the *serratus ventralis* can be explained in terms of chemical differences. The lower incidence of this condition in the *pectoralis profundus*, however, may be due to both chemical composition and chilling rate. The accelerated chilling rate may be due to thinness of muscle, anatomical location, and reduced anaerobic glycolysis. Further studies must be conducted to determine the influence of variations in chilling rate of

a particular muscle on the incidence of undesirable, soft, watery pork muscle characteristics.

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Biochemistry of Pork Muscle Structure. II. Preliminary Observations of Biopsy Samples versus Ultimate Muscle Structure[a]

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SUMMARY

Preliminary observations were made on biopsy and post-mortem samples from six Danish Landrace barrows. Additional studies were also conducted on post-mortem muscle samples from 30 Danish Landrace pigs of known breeding and nutrition. The biochemical characteristics studied were related to the continuous post-mortem measurements of pH and temperature, which were used to predict muscle structure. These data imply that the biopsy samples from pigs that ultimately showed the severe depression and elevation in pH pattern, contained greater total glycogen, lower percentage of acid-soluble glycogen, and faster phosphorylase activity. Similarly, these pigs possessed a smaller pyruvic acid pool and a greater lactic acid concentration. Results from measurements of methylene-blue reduction, in the presence of lactate, suggest that the methylene blue is reduced much more slowly in muscles from pigs with the type 4 pH pattern. Although this criterion was significantly correlated with zinc concentration, ultimate color intensity, and water-binding capacity, special emphasis was placed on the severe limitations that must be imposed on interpretation of this measurement. The significant correlations collectively suggest, however, that if the samples showed a rapid reduction of methylene blue, they were ultimately uniform in color, dry in appearance, and firm in structure. Significant additional correlations were shown for numerous biochemical characteristics of pork muscle tissue.

Numerous commercially important variations in the structure and chemical characteristics of muscle tissue have been reported (Callow, 1937; Dubowitz and Pearse, 1960; Bate-Smith, 1948; Bate-Smith and Bendall, 1956; Lawrie, 1953; Marsh, 1954; Hamm, 1955; Wismer-Pedersen, 1959a). Transitional appearances of pork muscle that range from pale, soft, exudative tissue to dark, firm, dry-appearing tissue have been described by Briskey *et al.* (1959a). The muscles were similar in physical characteristics at the time of slaughter; however, ultimately (24 hr), various types of muscle structure were manifest. These peculiarities seemed to be dependent, in part, on the quantity of glycogen metabolized, the rapidity and severity of pH decrease, and the elevation in pH during chilling (Briskey *et al.*, 1959a, b; Briskey, 1959a, b).

Lawrie *et al.* (1958) suggested that the development of pale, exudative tissue with abnormally low pH involves the extent and rapidity of the breakdown of adenosinetriphosphate. Wismer-Pedersen (1959a) also implied that these peculiarities in muscle structure arise from differences in phosphorylase activity and rate of pH decrease immediately post-mortem. More recently, Wismer-Pedersen and Briskey (1961) briefly reported continuous patterns of abnormally low pH depressions and elevations that were associated with an inferior structure.

Although this type of tissue has been noted in several breeds, it seemingly occurs at a higher incidence in certain breeds (Janicki and Walczak, 1954; Judge *et al.*, 1959). Similarly, its incidence can be re-

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duced by feeding iodinated casein (Ludvigsen, 1955), forced exercise (Briskey *et al.*, 1959b), electrical shock (Lewis *et al.*, 1959), and environmental change (Sayre *et al.*, 1959). This paper relates certain enzymatic activities and glycolytic component concentrations of biopsy samples to their ultimate pH pattern and structure.

EXPERIMENTAL

Experiment I. Variations in enzymatic activity and glycolytic component concentration of biopsy and post-mortem samples of longissimus dorsi. Six Danish Landrace barrows (15–20 kg) of known breeding were secured and subjected to a normal Danish feeding regimen. When the pigs weighed 60–70 kg they were anaesthetized with trichloroethylene, and biopsy samples (25 g) were secured from the lumbar region of the *longissimus dorsi* muscle of the right side, according to the procedure outlined by Merkel *et al.* (1954).

Physical and chemical determinations of biopsy samples. The pH values of the biopsy samples were determined with a Radiometer pH meter (Model 24). Portions of the samples were frozen in dry ice and subsequently analyzed for total glycogen by the procedure of Brand (1936); trichloroacetic-acid-soluble glycogen as outlined by Meyer and Herschberger (1957); lactic acid essentially as reported by Barker and Summerson (1941); and pyruvic acid as suggested by Friedemann and Haugen (1943). Fresh-tissue samples were analyzed for phosphorylase activity by the method of Sutherland and Cori (1951) as modified by Wismer-Pedersen (1959a). The results are expressed as mg of glycogen metabolized per g at 37° for 30 minutes. Tissue suspensions were prepared essentially as outlined by Bhagvat and Devi (1949). Five grams of biopsy tissue were weighed and ground in an ice-cold mortar with cold 0.1*N* phosphate buffer (pH 7.2). The suspensions were homogenized 1 min in a Tarax homogenizer during constant chilling with ice water. The homogenized solution was passed through a fine cloth and the residue homogenized again for 1 min. Final volumes were adjusted to contain 100 mg of wet tissue per ml of solution. The lactic dehydrogenase activity of the tissue suspensions was estimated by the modified method of Thunberg (Bhagvat and Devi, 1949). The results are expressed as μg of methylene blue reduced by 100 mg protein in the presence of lactate substrate during 1 hr. The activity of pyruvic oxidase was determined by the ability of the tissue to oxidize pyruvic acid *in vitro* at 37°C (Bhagvat and Devi, 1949). Two ml of the tissue suspension were incubated 1 hr with 100 mg of pyruvic acid at 37°C. The pyruvic acid remaining in the reaction mixture was estimated (Dubowitz and Pearse, 1960). The results are expressed as mg of pyruvic acid removed by 100 mg protein in 1 hour.

Physical and chemical determinations of post-mortem samples. Three weeks after removal of biopsy samples the pigs were slaughtered at the Roskilde Bacon Factory. Samples of the *longissimus dorsi* at the area of the 12th rib of the left side were secured for analysis as outlined for the biopsy samples. The carcasses were quickly transferred to the Danish Meat Research Institute and held at 3°C and 90% humidity for continuous recordings of pH and temperature in the *longissimus dorsi* of the left side at the area of the second lumbar vertebra (Wismer-Pedersen and Briskey, 1961). Samples were subsequently secured at the 2-hr and 24-hr periods for glycogen, lactic acid, and pyruvic acid, as described above. The samples were also analyzed for loose-water according to the procedure of Wismer-Pedersen (1959a), hematin by the method of Lawrie (1956), zinc as outlined by Sandell (1950), muscle classification by the method of Briskey *et al.* (1959a), and color intensity with the Elrepho Reflectance Meter (Zeiss, 1960) at 540 mμ. A standard of magnesium-oxide was used, with 100 as pure white.

Experiment II. Further studies to determine correlation coefficients between various constituents and structure. Thirty Danish Landrace pigs of known breeding from the Sjælland Testing Station were given a normal ration and slaughtered at the Roskilde Bacon Factory as they reached 88–89 kg. Within 15 minutes of bleeding, samples of the *psoas major* of the right side were removed and immediately analyzed for phosphorylase activity, and lactic dehydrogenase activity as described in experiment I. The pH values were simultaneously determined in both the *psoas major* and *longissimus dorsi* muscles according to the procedure of Wismer-Pedersen (1959b). After the carcasses were chilled for 24 hr, samples were taken from the second lumbar region of the *longissimus dorsi* and the *psoas major*. These sections were analyzed for zinc, hematin, loose-water, pH, color intensity, and muscle classification as described in experiment I. The data were analyzed for correlation coefficients by the method of Snedecor (1950).

RESULTS

Experiment I. Variations in certain enzymatic activities and glycolytic component concentrations of longissimus dorsi biopsy samples. The analytical data from the biopsy and post-mortem samples are summarized (Table 1) according to pH pattern (Wismer-Pedersen and Briskey, 1961). Major differences can be noted between the biopsy sample data of tissue ultimately showing the type 2 and

Table 1. Chemical and physical characteristics (biopsy and post-mortem samples).

Chemical and physical factors	Age of sample											
	Biopsy			15 min			2 hr			24 hr		
	pH pattern [a]			pH pattern			pH pattern			pH pattern		
	A Normal	B Rapid	C Sharp	A Normal	B Rapid	C Sharp	A Normal	B Rapid	C Sharp	A Normal	B Rapid	C Sharp
pH	6.68	6.47	6.63	6.40	6.60	6.18	6.07	5.46	5.30	5.58	5.50	5.59
Glycogen (mg/g)	4.02	4.71	7.45	3.61	9.94	6.10	1.18	3.30	1.31	1.07	0.81	0.26
TCA glycogen (% of total)	50	37	39	.....	.....	.....	.....	.....	.....	.....	.....	.....
Lactic acid (%)	0.29	0.77	0.52	0.62	0.62	0.88	1.17	1.18	1.26	1.16	1.14	1.12
Pyruvic acid (mg %)	2.80	1.12	1.13	2.69	1.99	3.20	0.70	0.11	0.28	0.13	0.04	0.18
Phosphorylase (mg glycogen/g/30 min)	11.38	14.36	15.50	13.48	17.30	15.40	.....	.....	.....	.....	.....	.....
Methylene-blue reduction (μg/100 mg protein/hr)	4.20	5.89	3.50	4.66	8.24	3.40	.....	.....	.....	.....	.....	.....
Pyruvic oxidase (mg pyruvic acid removed/100 mg protein/hr)	100.60	97.50	96.70	96.30	98.2	94.50	.....	.....	.....	.....	.....	.....
Loose-water number (mg expressible H_2O/g tissue)	.....	.....	.....	277	318	362	392	410	496	430	456	499
Color intensity (Elrepho Reflectance Units—540 mμ)	.....	.....	.....	.....	.....	.....	.....	.....	.....	21.6	20.3	28.9
Muscle structure (I, pale, two-toned; IV, dark)	.....	.....	.....	.....	.....	.....	.....	.....	.....	3.50	3.00	1.20
Hematin (mg/g)	.....	.....	.....	.....	.....	.....	.....	.....	.....	0.24	0.28	0.30
Zinc (mg/g N)	.....	.....	.....	.....	.....	.....	.....	.....	.....	0.60	0.70	0.53

[a] Post-mortem pH pattern as outlined by Wismer-Pedersen and Briskey (1961). A (normal): carcasses with type 2 pH pattern (a gradual decrease to about 5.7 at 8 hr, with an ultimate pH of 5.3–5.7). B (rapid): carcasses with type 3 pH pattern (a relatively rapid decrease to about 5.5 at 3 hr, with an ultimate pH of 5.3–5.6). C (sharp): carcasses with type 4 pH pattern (a sharp decrease to a pH of about 5.1 at 1½ hr, and a subsequent elevation to 5.3–5.6).

type 4 pH patterns. The former were lower in glycogen concentration, lactic acid concentration, and phosphorylase activity. Conversely, they were higher in percentage of acid-soluble glycogen, pyruvic acid concentration, methylene blue reduction, and pyruvic oxidase activity. It can also be noted that the biopsy sample data of tissue that ultimately showed the type 3 pH pattern were similar in magnitude to data from tissue with the type 4 pH pattern except that the former showed a faster reduction of methylene blue.

The data from the 15-min post-mortem samples were, likewise, relatively similar in magnitude to data from their respective biopsy samples. The greatest variations, in this respect, were noted for samples with the type 3 pH pattern. These variations were especially pronounced for the glycogen concentration, phosphorylase activity, and methylene-blue reduction. Conversely, the 2-hr data imply differences in pH and loose-water that were similar to data previously reported (Briskey and Wismer-Pedersen, 1961).

It can also be noted that the 24-hr samples were similar in ultimate pH values and lactic acid concentrations. The samples with type 4 pH patterns, however, contained greater quantities of loose-water and lower concentrations of zinc, and were paler in color and lower in muscle classification (structure). The lactic acid concentrations also appeared to show a slight decrease from the 2- to 24-hr period in the tissue with the type 4 pH pattern. Extensive studies with myosin and lactic acid at 40–30°C, however, have to date shown no evidence of lactate binding or disappearance during this pH elevation.

Experiment II. Further studies to determine correlation coefficients between various constituents and structure. Table 2 gives correlation coefficients showing the relationships between pH_1, methylene-blue reduction, zinc concentration, color intensity, phosphorylase activity, and loose-water quantity of the *longissimus dorsi* muscle. Highly significant correlations (P 0.01) were derived between pH_1 and color, pH_1 and loose-water, color and methylene-blue reduction, and color and loose-water. Similarly highly significant (P 0.01) multiple correlations were also shown for pH_1, methylene-blue reduction, and color intensity; pH_1, methylene blue reduction, and loose-water; color intensity, phosphorylase activity, and methylene-blue reduction. Significant correlations (P 0.05) were also noted between pH_1 and methylene-blue reduction, methylene-blue reduction and loose-water, and methylene-blue reduction and zinc concentration.

Table 2. Correlation coefficients of various biochemical characteristics of muscle structure.

Characteristic [a, b] correlation between:	Correlation coefficient
pH_1 and:	
methylene-blue reduction (μg methylene blue reduced/100 mg protein/hr; lactate substrate)	+.38*
zinc (mg/g N)	+.27
color (Elrepho Reflectance Units, 540 mμ)	−.64**
phosphorylase activity and methylene-blue reduction (mg glycogen metabolized/g/30 min)	.38*
methylene-blue reduction and loose-water	.70**
methylene-blue reduction and color	.63**
color and:	
methylene-blue reduction	−.64**
phosphorylase-activity and methylene-blue reduction	.64**
hematin (mg/g)	−.28
methylene-blue reduction and:	
loose-water	−.33*
zinc	+.38*
loose-water and:	
zinc	−0.19
phosphorylase	+0.01
pH_1	−0.66**
color	+0.58**

[a] age of sample at time of analysis: phosphorylase activity and methylene-blue reduction = 15 minutes; pH_1 = 40 minutes; color, loose-water, zinc, and hematin = 24 hours.
[b] muscle: *longissimus dorsi* and *psoas major*.

DISCUSSION

Consideration of differences in biopsy samples. The pH values of the biopsy samples were similar, regardless of the ultimate muscle structure. This is in agreement with previous reports (Briskey and Wismer-Pedersen, 1959a, b). The seemingly lower pH values in the samples that ultimately showed the type 3 pH pattern can be attributed to a more severe struggle during sampling (Bate-Smith, 1936), which also explains the high level of lactic acid in these samples.

The high glycogen concentration in the biopsy samples that ultimately showed the severe pH depression and elevation characteristic of the type 4 pH pattern is in agreement with the significant correlation reported by Briskey *et al.* (1960) for initial muscle glycogen and ultimate muscle classification (structure). It is realized that many

factors influence the amount of glycogen extracted by cold trichloroacetic acid. Among these factors are: severity and duration of extraction procedure (Bloom and Russell, 1955); fragmentation of cells during extraction; differences in extractability of glycogen variously located within the cell; size of glycogen molecule and protein binding (Stetten, Katzen and Stetten 1958). An additional factor in pork tissue is the possibility for an immediate post-mortem change in structure that might make it more or less readily extractable. The acid-soluble glycogen data (Table 1) represent a single rapid extraction. More exhaustive procedures extracted greater quantities of glycogen. In view of the many factors that may influence these data it is difficult to discuss the significance, if any, of the decreased amount extracted in type 3 and type 4 tissue. Although they might have contained the same quantity of total acid-soluble glycogen, the amount extracted by the procedures reported primarily demonstrates differences in the availability of the glycogen for rapid extraction. The quantities are in relative agreement with the 55% reported by Bloom and Russell (1955) for readily extractable glycogen. It is therefore suggested that the glycogen is either located differently in the cells of the muscle tissue with type 4 pH pattern or is bound to phosphorylase at the time of tissue death (Stetten *et al.*, 1958). Though further study is required before a conclusion can be drawn, it is tempting to point out the relationships in the related data, namely, low trichloroacetic-acid-soluble glycogen, high phosphorylase activity, and rapid and severe post-mortem pH depression. These differences, therefore, might be due to phosphorylase-glycogen binding (Stetten *et al.*, 1958) or inhomogeneity or inaccessibility, as suggested by Lawrie (1955).

The differences in phosphorylase activity, though not great, are in line with the suggestion of Wismer-Pedersen (1959a) that pigs with low pH_1 have a higher phosphorylase activity. As pointed out by Sutherland and Cori (1951), this can become an especially important factor in determining glycolytic rate. It is questionable in these particular samples, however, whether the phosphorylase activities in type 3 and 4 samples were varied enough to contribute to any change of pace in the glycolytic process.

The relatively high lactic acid concentration of these samples suggests that these animals were in a more excitable state, and secondly that this breakdown may have conceivably started prior to death, as intimated by Wismer-Pedersen (1959a). The pyruvic acid data imply that there were, in effect, larger pyruvic acid pools in the living tissue of normal pork musculature than in tissue that elicits a rapid pH fall.

Pyruvic oxidase activities showed only a slightly greater removal of pyruvic acid in the type 2 muscle. Therefore, it is not considered to be, in itself, a determinant of the amount of lactic acid formed prior to death in the tissue with the type 4 pH pattern. Conversely, the most marked differences appeared in the reduction of methylene blue (lactic dehydrogenase activity). Although this is a reported method for the determination of lactic dehydrogenase activity (Bhagvat and Devi, 1949; Kuznetsova *et al.*, 1953), it is known to be very sensitive to the oxygen content of the tissue, DPN–DPNH ratio, pH, denaturation, and other factors. It has, therefore, not been strictly discussed as dehydrogenase activity but rather as methylene-blue reduction, which would be influenced by and encompass the various factors. pH would seem to be discounted as a variable, since all biopsy samples were relatively uniform in this measurement. The rate-limiting velocity of this reversible enzyme in the forward direction is the rate of dissociation of the lactic dehydrogenase–DPNH complex (Winer and Schwert, 1958). However, the problem in this case is an accelerated pace in the forward direction and a possible reduced pace in the reverse direction. Although lactic dehydrogenase is not generally thought to be a pacemaker (Krebs, 1957), the oxygen tension or oxidation of DPN in association with it might have a pronounced influence on the lactic–pyruvic ratio (Husckabee, 1958). If it is viewed as a measure of lactic dehydrogenase activity it would support the recent suggestion of Dubowitz and Pearse (1960) that a high lactic dehydrogenase activity prevents all the lactic acid from going to the

liver for further oxidation. It is further interesting to note that the lactic dehydrogenase activity was also reduced in protein-depleted rats, in which the enzyme picture actually resembled that of a young rat (Ross and Ely, 1951). If this decreased lactic dehydrogenase activity actually occurs consistently in the pigs that are ultimately inferior in muscle structure, and if the inferior muscle structure occurs more readily in muscular pigs (Ludvigsen, 1955), then it could be construed to imply that maturation of the enzyme system has not kept pace with the growth of the muscle mass. In relation to Ross and Ellys' experiment it might be interesting to note that Hupka (1952, 1953) produced pork with exudative muscle tissue on a feeding regimen low in protein. This matter must be studied further, and will be of importance only if other procedures and more detailed studies continue to show differences in the enzyme activities of these tissues.

Chemical and physical variations in postmortem samples. The data for the 15-min samples were remarkably similar to their respective biopsy counterparts, except for phosphorylase activity and methylene-blue reduction. Since these differences were also noted in biopsy samples with comparable pH values, it does not appear that the pH variations in the 15-min samples accounted for the differences in these two characteristics. It should be emphasized that when phosphorylase activity and methylene-blue reduction were both especially high, a normal structure (type 3 pH pattern) prevailed. The hematin data support the reports of Lawrie (1956), Briskey *et al.* (1959b, 1960), and Wismer-Pedersen (1959a), that the differences in color and structure are not attributable solely to pigment concentration. Swift and Berman (1959) recently reported that zinc appeared to be highly correlated with pH and water retention in beef muscle. Since zinc is also a functional component of lactic dehydrogenase (Vallee and Wacker, 1956), it is interesting to note that the zinc concentrations were lowest in the tissue with the type 4 pH patterns, which were inferior in water retention, structure, color, and methylene-blue reduction. As pointed out by Underwood (1959) the total zinc content of a tissue gives no information on possible differences in its physiological availability or intracellular distribution—all of which could be important in connection with pH and water retention as well as lactic dehydrogenase activity. It can further be inferred from the data of Pfleiderer *et al.* (1958), that zinc is not likely to participate in the catalytic action of lactic dehydrogenase since, after treatment with agents forming zinc complexes, the enzymes retained all of the activity but no zinc.

Correlations between various biochemical characteristics of muscle structure. The fact that pH_1 is highly correlated to color (−0.64) and loose-water (−0.66) supports the pH_1 measurement as an effective guide to muscle structure. Since the multiple correlation for these factors and methylene-blue reduction does not change the magnitude of the correlation, the first three appear to be the most important. Similarly, the highly significant correlation of +0.58 between loose-water and color implies that muscles with large quantities of loose-water are inclined to be especially pale in color intensity. It is further interesting to note that methylene-blue reduction was significantly correlated with pH, color, loose-water, and zinc. The zinc content, however, was not correlated with pH or loose-water. This suggests that the methylene-blue reduction at the time of slaughter might, after study, be a reliable indication of muscles that will ultimately have inferior structure. Additionally, samples with an especially high reduction of methylene blue may be more inclined to an ultimately normal structure, color, and water-retention. From these observations it may be assumed that the development of the various patterns of the pH-time sequence, and consequently development of "watery" or normal tissue, is dependent on a number of factors. Among these factors are: 1) the extent of glycogen reserves in the muscle tissue at time of slaughter, 2) the phosphorylase activity and the state of glycogen in the tissue, and 3) the methylene-blue reduction activity of the tissue. The first two factors appear from previous experience to be related to the condition of the animal at the time of slaughter, whereas the third might conceivably be intrinsic in the development of the muscle enzyme system. One may find

support for this assumption in the significant correlation between the methylene-blue reduction activity and the zinc content. These results might thus infer that the structural development in pork muscle post-mortem is governed by an interaction between the sensitivity of the musculature and the state of the animal at time of slaughter.

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The Post-Mortem Oxygen Requirements of Bovine Tissue [a]

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SUMMARY

The activity of succinic dehydrogenase of post-rigor bovine tissue was determined at various temperatures and pH values. The oxygen uptake thus measured yielded typical curves. In the first 15 hr the uniform gas uptake can be accounted for by oxygenation of myoglobin, solution of oxygen and nitrogen, and enzymatic oxygen uptake. After 15 hr the oxygen uptake is due to enzymatic reaction. The enzymatic uptake of oxygen is about 0.7 μL/hr/g of wet tissue. The evolution of carbon dioxide was about 1.1 μL/hr/g of wet tissue. Tissue sample taken from deep in the musculature, however, showed a larger carbon dioxide evolution in the first 4 hr of exposure (before equilbrium is established) to an air atmosphere.

The myoglobin of muscle can bind oxygen to form oxymyoglobin or it can be oxidized by oxygen to form metmyoglobin. The reactions of myoglobin with oxygen are important in the over-all oxygen uptake by tissue. The oxygenation of myoglobin takes place on a mole-for-mole basis. Ginger *et al.* (1954) found that beef contains an average of 4 mg of myoglobin per g of wet tissue. On that basis about 5 μL of oxygen would be required to oxygenate the pigment in 1 g of muscle.

George and Stratmann (1952) established that the oxidation of myoglobin to metmyoglobin requires 2.5*M* oxygen. Oxidation of the myoglobin in 1 g of muscle to metmyoglobin would therefore require about 13 μL of oxygen if the reaction were carried to completion. Reducing conditions in the tissue can be such that an oxidation-reduction cycle could increase the requirement beyond 13 μL.

The surviving post-mortem respiratory enzyme systems in animal tissues have been investigated by Andrews *et al.* (1952) and Grant (1955). They showed the succinic dehydrogenase system to be the most stable of the post-mortem respiratory enzyme systems involving molecular oxygen.

When an animal dies and blood circulation ceases, the tissues can no longer exchange gases with their external environment. The net result is a depletion of the remaining oxygen in the tissue, along with a commensurate build-up of carbon dioxide. When the tissues become essentially devoid of oxygen, anaerobic metabolism results in the production of lactic acid and an ultimate pH of 5.8–5.6. The tissue will, upon exposure to air, achieve a steady state with respect to the gases present in the atmosphere and the temperature of the tissue.

When compared to the oxygen utilized by the succinic dehydrogenase system, the oxidation of other tissue constituents, such as fat, is of minor consideration in the post-mortem state. It therefore becomes unnecessary to account for these minor oxidative systems in bovine tissue.

The present investigation was conducted to establish the oxygen requirements of post-mortem bovine tissue as well as to ascertain the various factors influencing this requirement.

EXPERIMENTAL PROCEDURE

All samples were taken from the *semimembranosus, adductor,* and *biceps femoris* muscles of the bovine round. The muscles were all post-rigor.

Succinic dehydrogenase activity was determined manometrically by the procedure of Schneider and Potter (1943). Tissues were homogenized for 1 minute in a Waring blender by the method of Marquette and Schweigert (1950).

Oxygen uptake of freshly cut beef was determined manometrically in 100-ml Warburg flasks. Samples of muscle were cut into cylinders having a diameter of 38 mm and a height of 36 mm. To provide two identical meat surfaces, two glass

[a] Journal paper No. 209 from the American Meat Institute Foundation.

sample cups, of 38-mm inside diameter and 18-mm height, were placed over the ends of the muscle and a cut was made through the center of the sample. The cut samples were quickly placed in the flasks, attached to the manometers, and placed in a constant-temperature bath. Manometer changes were recorded immediately, with no time allowed for equilibration. To minimize temperature effects, samples were stored 24 hr before the experiment at 1°C. The Warburg apparatus was also placed in cold rooms to further assist constant-temperature conditions. Experiments to determine oxygen uptake and carbon dioxide evolution of bovine tissue were conducted at 1°C. For estimation of the actual amount of sample exposed to the atmosphere, the oxygen penetration depth of each sample was measured. Penetration depth was readily determined by measuring the distance from the sample surface to the point of formation of the metmyoglobin band. This band in beef forms at a depth of a few mm to about 16 mm after 4–7 days' storage, depending on the temperature of the tissue and the per cent of oxygen in the atmosphere.

RESULTS AND DISCUSSION

The effect of temperature and pH on the succinic dehydrogenase system of bovine tissue. As seen in Fig 1, temperature provides an excellent means of controlling the enzymatic demands of bovine tissue for oxygen. The oxygen uptake of the succinic dehydrogenase system was measured in phosphate buffers at various temperatures and pH values of 6.4, 7.4, and 8.0. Each point is the average of six measurements at each pH and temperature given. As the temperature approached 10°C oxygen uptake at the three different pH values tended to converge.

Oxygen uptake of bovine tissue. It was observed in the early stages of this work that the oxygen uptake for samples taken on consecutive days seemed to give different initial uptake values. Samples were then placed in reaction flasks with and without potassium hydroxide in order to determine the carbon dioxide exchange of the tissue. During the first few hours in the reaction flask, the samples evolved considerable carbon dioxide (Fig 2). Each point shown on this curve is the average of 30 determinations. The reasons for the build-up of carbon dioxide become apparent when the post-mortem changes are considered. The pH of

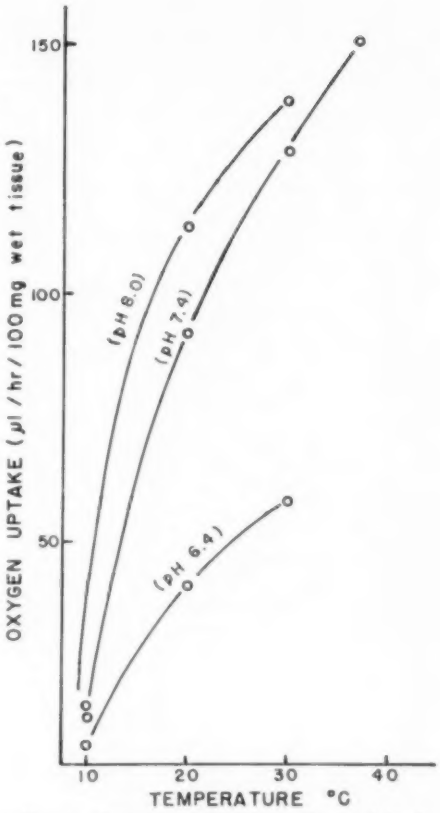

Fig. 1. Oxygen uptake of muscle succinic dehydrogenase as a function of temperature and pH.

normal living tissue is 7.2–7.4, and the post-mortem pH is in the range of 5.6–5.8. The

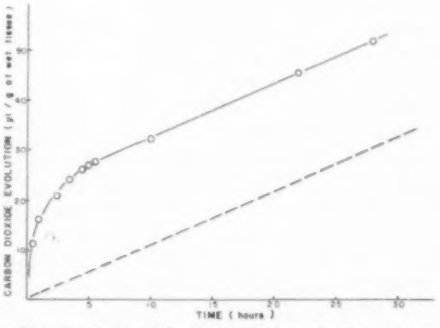

Fig. 2. Carbon dioxide evolution of post-mortem bovine tissue at 1°C.

ratio of bicarbonate to carbon dioxide would be about 15 : 1 at pH 7.3, and 1 : 2.5 at pH 5.7. The tissues cannot exchange gases with their environment once circulation ceases. When the muscle is cut, however, gas exchange with air is again possible until a steady-state condition is achieved. These samples were all freshly cut and taken from deep in the musculature; however, if a sample of about 3 in. thickness is taken off and allowed to remain unwrapped in the refrigerator for 24 hr, the amount of carbon dioxide evolved after the sample is placed in the manometer is very noticeably decreased. The resulting curve shows no large upsurge, but is rather a straight-line function with time, as shown by the dotted line in Fig. 2. Fig. 3 gives the oxygen uptake of bovine tissue; each point on the curve is the

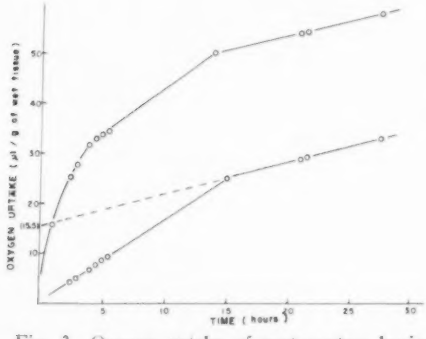

Fig. 3. Oxygen uptake of post-mortem bovine tissue at 1°C. Upper curve uncorrected for CO_2 evolution; lower curve corrected for CO_2 evolution or O_2 uptake for sample stored for 24 hr before determination.

average of 30 determinations. The linear relationship shown by the dotted line in Fig. 3 resulted when the carbon dioxide evolution for the first 4 hr was subtracted from oxygen uptake for the same period. The initial carbon dioxide evolution causes a decrease in sample volume, much the same as a loss of carbon dioxide would cause a decrease in the volume of leavened dough. In the determination of oxygen uptake this sample volume decrease causes the manometer to show a larger volume change than is actually accounted for by the oxygen uptake. It therefore becomes necessary to correct the oxygen uptake curve for the carbon dioxide evolution during the period when the sample volume is decreasing. If samples 3 in. thick, as described previously, are allowed to remain unwrapped in a cooler for 24 hr before introduction in the reaction flask, oxygen uptake is a straight-line function with time, as shown by the dotted line in Fig. 3. The corrected curve thus shown in Fig. 3 is typical of tissue oxygen uptake, and the slopes vary with the amounts of myoglobin and respiratory enzymes present and the temperature of the tissue.

Uptake of oxygen accounted for by oxygenation of myoglobin and resaturation of tissue fluid with oxygen. An important consideration in an estimation of oxygen uptake due to myoglobin and tissue fluid saturation is the amount of sample exposed to the air atmosphere by the cutting of a fresh surface. As mentioned previously, the post-mortem tissue rapidly becomes depleted of oxygen. The water in the tissue will hold more dissolved oxygen at 1°C than at a normal bovine body temperature (39°C). As the oxygen dissolves in the tissue surface it tends to diffuse into the tissue. Theoretically, a gas would diffuse through the tissue until saturation is complete. This is not the case, however, when oxygen is being utilized in the tissue for oxidations. We may consider that a certain portion is utilized as the oxygen penetrates the tissue, so that the amount available for further diffusion into the tissue is being constantly decreased. Thus, the ultimate depth to which oxygen will penetrate will be a function of the enzymatic demands and the amount dissolved in the tissue. The rate of diffusion of oxygen is also a limiting function of penetration of the tissues. An increase in temperature increases the rate of diffusion and the enzymatic demands, whereas the solubility of oxygen is decreased. The net effect of a temperature increase of the tissue is a decrease of penetration depth, which results in less tissue being exposed to the air environment for a given surface area. The oxygen uptake of bovine tissue changes slope after about 15 hr since the tissue fluids and myoglobin are now at equilibrium with the surrounding atmosphere. The slope of the curve after 15 hr is, in all probability, a

measure of oxygen uptake due to enzymatic reactions. If the enzymatic slope is extrapolated to zero time, its intercept should be oxygen uptake due to tissue fluid and myoglobin saturation as well as an effect due to nitrogen uptake. The nitrogen uptake is due to the difference between the temperature of the living animal (39°C) and that of the cooled tissue (1°C). The extrapolated value obtained from this work is about 15.5 μL of fulfilled gas uptake. Since the number of samples used was large, a theoretical fulfilled gas uptake value can be calculated from average figures. Assuming 4 mg of myoglobin per g of tissue and an average molecular weight of 17,000 for myoglobin, it can be calculated that oxygen uptake due to myoglobin oxygenation is 5.27 μL/g of wet tissue. For the oxygen dissolved in the aqueous phase of the tissue, a figure for oxygen solubility in water can be used as well as the fact that the moisture content is about 70% and air contains 0.2 atmosphere of oxygen. (It is also necessary to consider that the dissolved oxygen is only one-half of this value since oxygen in a sample is between the limits of 100% at the surface and 0% at the point of maximum penetration.) Therefore, the oxygen uptake that is accounted for by the solution of oxygen is 3.42 μL/g of tissue. The amount of nitrogen that will be taken up by the tissue can be calculated from the difference in solubility at the experimental temperature (1°C). Using a moisture figure of 70% and 0.8 atmosphere nitrogen pressure, the nitrogen uptake is calculated to be 6.55 μL. The sum of the uptake due to oxygenation of myoglobin, and solution of oxygen and nitrogen, is 15.24 μL. Since the extrapolated value of 15.5 μL is in such good agreement with the theoretical value of 15.24 μL, the validity of the methods used appears well substantiated. The gas uptake shown in Fig. 3, in the lower curve from zero to about 15 hours, is accounted for by the oxygenation of myoglobin, the solution of nitrogen and oxygen, and enzymatic uptake of oxygen. The curve beyond 15 hours is a measure of enzymatic oxygen uptake. Therefore, the enzymatic uptake of oxygen is about 0.7 μL/hr/g of wet tissue. The carbon dioxide evolution is about 1.1 μL/hr/g of wet tissue.

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The Anthocyanins of the American Cranberry [a]

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SUMMARY

The red pigments from Early Black cranberries were extracted, separated, and purified. Four distinct bands appeared on silicic acid column. The slowest-moving band was identified as cyanidin 3-monogalactoside, the next was peonidin 3-monogalactoside. The third and fourth were respectively glycosides of cyanidin and peonidin. The absorption coefficients of the purified pigments and the aglucone portions were determined in 95% ethanol–0.1*N* hydrochloric acid (85:15 v/v).

Willstaetter and Mallison (1915) characterized one red pigment from the English cowberry (*Vaccinium vitis idaea* L.) as 3-beta-galactosidyl cyanidin. This plant has been called the European cranberry and is close relative of the North American cranberries. Grove and Robinson (1931) identified one pigment as 3-beta-glucosidyl peonidin from berries from Newfoundland and Cape Cod that they identified as *Oxycoccus macrocarpus* Pers. Markakis (1954), using paper chromatography, identified one pigment in Cape Cod cranberries as cyanidin monogalactoside.

The present work was undertaken to clarify the identity of the red pigments in cranberries in the hope that a simple ratio method based on absorption data of the actual pigments could be developed to replace the Congo Red method (Francis, 1957) for production (Francis and Atwood, 1961) and processing studies (Servadio and Francis, 1961). It is now apparent, however, from the complexity of the pigment mixture and the similarity of the absorption spectra, that any analytical method for determination of each pigment must depend on separation of the individual pigments.

MATERIALS AND METHODS

Sources of berries. The cranberries used in this work (*Vaccinium macrocarpon* Ait., variety Early Black) (Chandler and Demoranville, 1958) were harvested in the fall of 1959 and stored fresh under refrigeration until required.

Extraction and purification of crude pigment. Five-hundred-gram samples of the fresh berries were comminuted in a Waring blender with 400 ml of *n*-butanol for 3 minutes. After standing overnight, the liquid was filtered off and the residue extracted with a second 400-ml portion of *n*-butanol. After standing overnight, the liquid was filtered off and combined with the first extraction in a separatory funnel. The water phase was removed, and 1500 ml of petroleum ether was added to the butanol phase (about 750 ml). A lower aqueous phase separated, which contained nearly all the red pigments. The aqueous phase was purified by passage through a resin column (Amberlite CG-50, Type I, Rohm & Haas Inc., Philadelphia, U.S.A.; the resin was packed in 5 × 28-cm columns and washed with distilled water before use; used columns were regenerated by washing with distilled water, then dilute hydrochloric acid, and finally distilled water again). The red pigments were retained on the column and a brownish pigment passed through. After washing with distilled water until the eluate was clear, the pigments were readily eluted with 95% ethyl alcohol. The ethanolic extract was evaporated to dryness under vacuum, and the residue redissolved in anhydrous methanol for ease of collection, and allowed to dry at room temperature. The amorphous residue labeled "crude pigment," was obtained in yields of about 350 mg from 500 g of fresh berries.

Separation of individual pigments. The crude pigment was purified by passage through a column of silicic acid (100 mesh, Mallinckrodt Chemical Works). Spaeth and Rosenblatt (1950) used silicic acid to separate mixtures of synthetic anthocyanidins and Li and Wagenknecht (1955, 1958) later used a somewhat modified method to separate

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cherry anthocyanins. The silicic acid columns proved very useful for separating cranberry anthocyanins when used with the upper phase of a mixture of *n*-butanol-acetic acid-water (B.A.W.) and benzol (4:1:5:2 v/v). The crude pigment was dissolved in a minimum amount of 2% hydrochloric acid in methanol and placed on the column prior to development with the above solvent. A 5 × 40-cm column prepared from 350 g of silicic acid and 193 ml of distilled water would separate about 100 mg of crude pigment into four distinct fractions and several other bands, present in very small amounts. One disadvantage of this column was the time required (7 days) to get a good separation with 100 mg of pigment.

The fractions were collected as they came off the column after discarding the leading and trailing edge of each band. After evaporating to dryness under reduced pressure in the cold, each pigment was taken up in a small amount of methanol and evaporated to dryness. The amorphous precipitates were collected and labeled Bands I to IV, from the slowest-moving bands to the most rapid. Bands I and III were reddish-violet, and Bands II and IV were reddish-pink.

Paper chromatography of the individual pigments. Samples of the pigments after purification on the silicic acid were dissolved in 1% hydrochloric acid in methanol and developed on Whatman No. 1 paper chromatograms by the descending method. Data on the R_f values for four solvents are presented in Table 1.

Table 1. R_f values of cranberry anthocyanins.

Sample	HClHAc [a]	1% HCl [b]	Bu HCl [c]	B.A.W.[d]
Band 1	0.25	0.06	0.26	0.31
Band 2	0.29	0.07	0.30	0.35
Band 3	0.24	0.05	0.38	0.40
Band 4	0.28	0.06	0.45	0.50

[a] Water-acetic acid-12*N* hydrochloric acid, 82:15:3 (v/v).
[b] Water-12*N* hydrochloric acid, 97:3.
[c] *n*-butanol-2*N* hydrochloric acid, 1:1.
[d] *n*-butanol-acetic acid-water, 4:1:5.

The aglycones were prepared by stirring a 5–20-mg sample of each purified pigment with 2 ml of 5% hydrochloric acid in water. After 15 min in a boiling water bath, the tubes were cooled and a portion of the solutions evaporated to dryness. The residues were taken up in 2% hydrochloric acid in methanol and spotted on the paper in the same manner as the anthocyanins. Chromatographed at the same time was an authentic sample of cyanidin (obtained through the courtesy of Dr. L. Jurd, Western Regional Research Lab., Albany, California). The data are presented in Table 2.

Table 2. R_f values of cranberry anthocyanidins.

Sample	Forestal [a]	B.A.W.[b]
Band 1	0.50	0.67
Band 2	0.64	0.70
Band 3	0.49	0.66
Band 4	0.63	0.69
Cyanidin	0.49	
Peonidin	0.63	

[a] Water-acetic acid-12*N* hydrochloric acid, 10:30:3.
[b] *n*-butanol-acetic acid-water, 4:1:5.

A sample of peonidin was extracted from red peonies by the following procedure: 50 g of petals were blended with 100 ml of *n*-butanol and allowed to stand 1 hr. After filtering, 2 volumes of petroleum ether were added and a lower aqueous phase separated. Chromatography of the aqueous phase in Forestal solvent showed a major component with the characteristics of peonin, and a minor component. The aqueous phase was hydrolyzed by the addition of 20 ml of 15% hydrochloric acid and heating 5 min in a boiling water bath. After cooling, the precipitate was filtered off. The clear filtrate was allowed to stand for several days in the cold, and red crystals appeared. After washing and drying, the crystals were chromatographed and showed only one band. The pigment was presumed to be peonidin. This pigment was chromatographed with the others, and the data are presented in Table 2.

Identification of sugar moieties. A portion of the hydrolysis solution prepared for the aglycones was used to recover the sugar moieties. A sample of the solution was shaken with three portions of isoamyl alcohol until the hydrolysate was almost colorless and then passed through an ion-exchange column (1.2 × 35-cm columns prepared from Amberlite 1R-4B(OH) resin; Rohm & Haas Company, Philadelphia, Pennsylvania) for deacidification. The eluates were concentrated under reduced pressure and chromatographed according to the procedure of Jermyn and Isherwood (1949). The ethyl acetate-acetic acid-water (3:1:3 v/v) system was used to give an adequate separation between glucose and galactose.

The R_f values for the sugars are presented in Table 3. The sugar moiety from Bands 1 and 2 was galactose. The sugars from Bands 3 and 4 have not yet been identified, but they are not glucose or galactose. Spots comparable to xylose were detected in the chromatograms, but excessive tailing of the chromatograms prevented positive identification. Identification of these sugars must await further purification of Bands 3 and 4.

The data for the aglycones (Table 2) indicate that Bands 1 and 3 contain cyanidin, and Bands 2

Table 3. R_f values of sugar moieties of cranberry anthocyanins.

Sample	Ethyl acetate–water–acetic acid (3:1:3 v/v)	Butanol–acetic acid–water (4:1:5 v/v)
Glucose	0.28	0.18
Galactose	0.25	0.13
Band 1 hydrolysate	0.25	0.13
Band 2 hydrolysate	0.25	0.13

and 4 contain peonidin. This conclusion was supported by the results of a series of color tests with sodium hydroxide, ferric chloride, sodium acetate, and aluminum chloride (Gilman, 1938; Harborne, 1958). In all cases, the aglycones from Bands 1 and 3 were identical, as were also those from 2 and 4. Comparison of the pigments with authentic cyanidin and peonidin provided further evidence of their identity.

The data for the anthocyanins (Table 1), when compared with the R_f values supplied by Harborne (1958), indicated that all four pigments were monoglycosides. The R_f values for HClHAc, 1% HCl, and BuHCl suggest that Bands 1 and 2 are respectively cyanidin monogalactoside and peonidin monogalactoside, but the values supplied by Harborne for the two pigments in B.A.W. were slightly higher than those indicated here. (Values for peonidin monogalactoside were not supplied by Harborne, but were assumed by interpolation and analogy with similar pigments.) Furthermore, since all four pigments showed no fluorescence under ultraviolet light, the sugar residues are probably attached in the 3-position. The sugars are found, in nearly every case, in the 3- or 5-position, and the 5-monoglycosides are fluorescent.

The data in Table 1 for the R_f values of Bands 3 and 4 indicate that the anthocyanin must be more soluble in the butanol mixtures and less soluble in the aqueous solvents. A xylose sugar moiety would fit these criteria, but confirmation must await further pigment purification.

Absorption spectra. Purified samples from the silicic acid column were dried overnight in a desiccator over both phosphorus pentoxide and concentrated sulfuric acid. The dry samples were weighed on a microbalance, dissolved in a mixture of ethanol and hydrochloric acid (85:15 95% ETOH, 0.1*N* HCl) and measured in a Beckman DU spectrophotometer. Absorption spectra from 225 to 625 mμ were measured, and the absorption coefficient calculated at the wavelength of maximum absorption. The data are presented in Table 4.

Attempts were made to crystallize the pigments from the chromatographically pure fractions. The pigments were dissolved in methanol containing 2% hydrochloric acid and allowed to evaporate slowly in the cold. Band 2 was the only fraction that yielded crystals readily. The fine lustrous crystals were filtered off, washed, and dried. The pigment in Band 1 would not crystallize readily; therefore it was purified by precipitation with saturated picric acid. The picrate readily separated in the cold, and, after filtering and washing, the picrate crystals were dissolved in ethanol containing 5% hydrochloric acid. After filtering, the amorphous precipitate was precipitated by addition of ethyl ether, dried, and dissolved in the ethanol–hydrochloric acid reagent for optical-density determinations.

It is apparent from the data in Table 4 that the crystallization and picrate precipitation raised the absorption coefficients of the anthocyanin pigments considerably. The silicic acid chromatography produced pigments that were quite adequate for paper chromatography but were not pure enough for determination of absorption coefficients. The values for Bands 3 and 4 were very low, indicating that some impurities were probably present. This

Table 4. Absorption coefficients [a] of cranberry pigments.

	Band 1	Band 2	Band 3	Band 4
Anthocyanins				
(a) Purified by column chromatography	$E_{535} = 511$	$E_{532} = 527$	$E_{526} = 334$	$E_{532} = 422$
(b) Purified by crystallization from (a)	.....	$E_{532} = 935$	.....	.....
(c) Purified by picrate precipitation from (a)	$E_{535} = 894$	.....	.....	.....
Anthocyanidins				
From (b) above	.....	$E_{530} = 1150$	.....	.....
From (a) above	.....	.....	$E_{515} = 1134$	.....

[a] Absorption coefficients expressed as E, 1%, 1 cm, at the wavelength of maximum absorption.

may explain some of the difficulty with tailing in identification of the sugar moiety.

Absorption data on the anthocyanidins prepared from the purified pigments are also presented in Table 4.

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Studies on Domestic Dates. II. Some Chemical Changes Associated with Deterioration[a, b]

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SUMMARY

Some chemical changes in Deglet Noor dates during storage at 49° C were studied. Darkening is the combined result of both oxidative and nonoxidative browning. The former, which constitutes about 10–20% of the total darkening, can be inhibited by storage in inert gas atmospheres. The decrease in pH of the tissue is primarily the result of nonoxidative nonenzymic reactions. Sucrose inversion, the result of invertase action, can be prevented by a mild heat treatment. Oxygen absorption and carbon dioxide production are due to both enzymic and nonenzymic reactions. Apparent phenolase activity increased during storage, whereas apparent peroxidase activity decreased.

Chemical and physical changes in domestic dates after harvest lead to their eventual deterioration. Insect infestation and microbial spoilage are controlled with fumigants and moisture adjustment. Blanching and/or sulfuring is used with most other dried fruits to retard deterioration; however, neither of these practices is presently used on dates. Refrigeration or freezing is useful in slowing deteriorative reactions during storage, but these methods do not offer protection during the subsequent prolonged periods when dates are exposed to room temperatures. There is need, therefore, for a process that will stabilize dates against deterioration.

Knowledge of the chemical systems responsible for deterioration of dates would be extremely helpful in developing methods of preservation. Previous workers (Rygg, 1956, 1957; Rygg *et al.*, 1953) showed that pronounced chemical and physical changes are associated with deterioration. These included an increase in darkness, softness, and sirupiness, and a decrease in pH, aroma, and flavor. Little is known concerning the chemical systems responsible for these changes. Nielsen *et al.* (1950) reported that darkening of pasteurized dates is primarily an oxidative reaction involving the tannins; unheated dates were not studied. Rashid (1950) demonstrated in dates a phenolase having the properties of a laccase, and suggested that both enzymic and nonenzymic browning may occur. The causes of the other changes associated with deterioration have not been studied.

This paper reports measurement of the rates of several chemical and physical changes that occur during the deterioration of dates. Also determined were the effects of a chemical preservative, inert gases, and heat treatments on the rates of these changes. Conclusions are made concerning the chemical systems responsible for darkening, pH decrease, sucrose inversion, oxygen absorption, and carbon dioxide production.

EXPERIMENTAL

Analytical methods. Darkness was measured by the reflectance and soluble dark pigment methods reported previously (Maier and Schiller, 1960). Sugars were freed from the date tissue by Soxhlet extraction with 80% ethanol, and the extracts were clarified with neutral lead acetate, disodium phosphate, and charcoal (Joslyn, 1950). Reducing sugars were determined by the Hassid

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[b] This investigation was supported in part by the Date Administrative Committee, Indio, California.

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ferricyanide method (Hassid, 1936); the results are reported as glucose on a dry-weight basis. Total sugars were also determined by this method after acid hydrolysis (Assoc. Offic. Agr. Chemists, 1945). Sucrose was determined, using the appropriate calculation, as the difference between total sugars and reducing sugars (Sinclair *et al.*, 1941). The pH of the ground tissue was measured by inserting the electrodes of a pH meter into the soft date paste. Moisture content was determined by drying for 20 hours at 70°C and 10 mm Hg. Analysis of the headspace gas in the jars for oxygen and carbon dioxide was performed with a paramagnetic oxygen analyzer. A Warburg respirometer was also used to study the gas exchange of dates at 49°C. Flasks of 145-ml capacity that could accommodate two intact dates (21 g) were used. In the study of seed respiration, 8 seeds weighing 6.33 g were used per flask. In all cases the flasks were gassed for 10 minutes with either air or pure nitrogen. Oxygen absorption was measured in flasks containing 0.4 ml of 20% KOH and a piece of fluted filter paper in the well to remove carbon dioxide. Carbon dioxide production was determined by standard Warburg calculations from gas exchange data of flasks containing no KOH and the known oxygen uptake of companion flasks. Rates were taken from the slopes of the straight-line portions of plots of time versus gas exchange.

Phenolase and peroxidase activities were assayed by a spectrophotometric method. Tissue extracts were prepared by homogenizing 5 g of ground dates and 20 ml of ice water and filtering, under refrigeration, through Whatman No. 1 paper. The substrate for phenolase was 0.05*M* in catechol and 0.1*M* in phosphate buffer, pH 6.0. That for peroxidase was 0.002*M* in guaiacol, 0.0005*M* in hydrogen peroxide, and 0.1*M* in phosphate buffer, pH 6.0. Activities were determined by placing 3.0 ml of substrate in a spectrophotometer cuvette, balancing the instrument, and then rapidly pipetting 0.60 ml of enzyme extract into the cuvette. After thorough mixing, absorbance measurements were made at 400 mμ for peroxidase and 410 mμ for phenolase at 10- or 15-second intervals. Plots of absorbance versus time gave straight lines. Initial rates, reported in terms of increase in absorbance per minute, were used as a measure of the activities at 25°C.

Experimental procedure. Choice natural Deglet Noor dates of 19.1% moisture content were used throughout this study. They were color-graded and thoroughly randomized to ensure sample uniformity. Two experiments were conducted. The first preceded the second by two months. During this time the dates for the latter experiment were kept in frozen storage (−18°C). About 25 dates (275 g) were weighed into each of seventy 967-ml glass jars. In the first experiment, two series of 7 jars each were used as untreated controls. Three series were regassed with nitrogen, prior to which 5 dates in each jar were injected with 0.25 ml of a solution containing 2 mg/ml of a commercial glucose oxidase-catalase mixture. In addition, two series were treated as stated above, sealed, then heated, one for 40 min (short heat) and the other for 60 min (long heat). Then the injected dates were removed and the jars regassed with air. The dates heated 40 min retained 78% of their original phenolase activity, whereas those heated 60 min gave negative phenolase and peroxidase tests. The heat treatment was carried out in a laboratory autoclave at 16 lb pressure (122°C). In the second experiment, one series was used as a control, a second was regassed with carbon monoxide, and a third was treated with 0.5 ml per jar of a preservative (85% methyl formate and 15% ethylene oxide) prior to sealing.

The gassing procedure was as follows: several drops of solder were fused to the lid of the jar, which was then pierced with a small hole. After the lid was firmly fitted to the jar it was evacuated (125 mm Hg) for one minute and then released with oxygen-free nitrogen or carbon monoxide. This process was repeated three times, and the hole immediately sealed with a soldering iron.

All jars were held at 49°C in a constant-temperature water bath for varying periods up to 618 hours. The jars were removed from the bath, and headspace gas analyzed as soon as they reached room temperature. The dates were then pitted and thoroughly ground to ensure sample uniformity before subsequent analyses.

RESULTS AND DISCUSSION

Effect of treatments on reflectance decrease. The results in Table 1 were obtained from graphs in which logarithm per cent reflectance was plotted against time in hours. The use of logarithm per cent reflectance gives more nearly linear curves (concave to the time axis) which are easier to fit with smooth lines. Time required for reflectance to change from 10 to 6% was chosen as a measure of the rate of darkening and is reported in units of decrease in per cent reflectance/100 hr. Previous work (Maier and Schiller, 1960) demonstrated that dates having a reflectance of 6% by this method were considered to be objectionably dark. Above 10% reflectance, data are sometimes irregular. Thus, the range between 10 and 6% reflectance offers consistent rates and

Table 1. Effect of treatments on reflectance decrease.

Treatment	Reflectance (%) Initial	600 hr at 49°C	Rate, (Δ% R/100 hr)	Per cent of control rate
Experiment 1				
Air [a]	16.8	4.35	1.86	100
Nitrogen [b]	16.8	4.85	1.49	80.1
Short heat	14.2	4.20	1.74	93.5
Long heat	8.04	3.20	1.97 [c]	105
Experiment 2				
Air	16.0	3.80	2.00	100
Carbon monoxide	16.0	4.25	1.68	84.5
Preservative	16.0	4.55	1.70	85.0

[a] Average of duplicates.
[b] Average of triplicates.
[c] This value is for the 8–6% reflectance range extrapolated to the 10–6% range.

covers the critical region of darkness with respect to appearance.

It is apparent from these data that darkening of dates under these conditions proceeds by two different pathways. The primary pathway at 49°C is nonoxidative and nonenzymic. This is shown by the failure of inert atmospheres and thermal enzyme inactivation to prevent the major part of darkening. The existence of a secondary oxidative browning pathway is indicated by the 15–20% reduction in rate of darkening in inert atmospheres. It is not clear whether the oxidative browning is catalyzed by enzymes. The 6.5% reduction in rate of the short-heat sample is in qualitative agreement with its 22% lower phenolase activity. It is difficult, however, to evaluate this small difference in rate, since experimental error approached this magnitude. The long-heat dates, in which phenolase and peroxidase activities were zero, darkened slightly faster than the unheated dates in air. This would seem to indicate that oxidative browning of unheated dates is nonenzymic. However, the complete inactivation of enzymes by the long-heat treatment was accompanied by considerable darkening. It is possible that browning precursors formed during the long-heat treatment caused an acceleration of subsequent nonenzymic browning sufficient to obscure inhibition of enzymic browning. The 15% reduction in rate of darkening of preservative-treated dates is thought to be a result of their lower pH. Qualitative tests in which the pH of dates was lowered with acetic acid indicated that the rate of darkening was slower in the treated fruit.

Effect of treatments on soluble dark pigment production. Extractable dark pigment concentration is another measure of darkening in dates (Maier and Schiller, 1960). The data in Table 2, obtained from

Table 2. Effect of treatments on soluble dark pigment production.

Treatment	Soluble pigment (mg/g) Initial	600 hr at 49°C	Rate (mg/g/100 hr)	Per cent of control rate
Experiment 1				
Air [a]	4.94	31.5	4.43	100
Nitrogen [b]	4.94	27.5	3.76	84.8
Short heat	6.64	29.3	3.78	85.5
Long heat	14.2	37.0	3.80	85.8
Experiment 2				
Air	4.94	30.8	4.31	100
Carbon monoxide	4.94	31.0	4.34	101
Preservative	4.94	34.8	4.98	115

[a] Average of duplicates.
[b] Average of triplicates.

graphs of soluble pigment concentration versus time, are given in mg pigment/g dry tissue. The data traced smooth curves concave to the time axis. Increase in pigment concentration in 600 hours was arbitrarily used as a measure of the rate of darkening, which is reported in units of mg/g/100 hr. The significance of the rate data is supported by the excellent agreement between the rates of air samples in Experiments 1 and 2 and by the fact that the greatest deviation observed between replicates in Experiment 1 was ±6.0%.

These results are in general agreement with the reflectance data in showing the existence of a primary nonenzymic nonoxidative browning pathway and a secondary oxidative pathway. The closely similar retardation in the rates of darkening caused by heat treatment or nitrogen storage suggests that an enzymic oxidative reaction system may be affected in each case. The reflectance and soluble pigment results for

darkening of the short-heat dates agree qualitatively and support this conclusion. The results for the long-heat dates disagree, however; reflectance shows an increased rate, and soluble pigment a decreased rate. Therefore, it appears that the long-heat treatment has an influence on subsequent browning reactions that makes impossible any direct comparison with unheated dates for purposes of determining whether oxidative browning is enzymic. In view of the relectance data, the failure of carbon monoxide to retard the rate of darkening and the accelerated rate of the preservative-treated sample may be attributed to increased pigment extractability. This might be the result of increased tissue breakdown caused by these chemicals.

These results show that both oxidative and nonoxidative browning reactions are involved in the darkening of unheated dates, and in addition, define the quantitative relationship between the two types of browning under the conditions studied. The presence of amino acids (Grobbelaar *et al.*, 1955; Rinderknecht, 1959) and reducing sugars suggests carbonyl-amine browning as the nonoxidative pathway. The requirement for oxygen and the presence of phenolase and polyphenols (Maier and Schiller, 1959; Rashid, 1950) suggests that the oxidative browning of unheated dates may be enzyme-catalyzed. Nielsen *et al.* (1950) found that the darkening of pasteurized dates is primarily an oxidative reaction. The relatively greater amount of oxidative than of nonoxidative darkening shown by their data may be due to the much higher moisture content (30%) of the fruit studied, the lower storage temperature, or the pasteurization process.

Effect of treatments on pH decrease. Rygg *et al.* (1953) first observed that a decrease in the pH of date tissue was associated with deterioration. As shown in Table 3, pronounced decreases in pH were also observed in the present work. Graphs of logarithm pH versus time gave smooth curves slightly concave to the time axis. The time required for the pH to change from 5.50 to 5.00 was arbitrarily used as a measure of the rate and is reported as pH decrease/100 hours (Table 3). This range covers the critical period during which the dates become unacceptable and closely corresponds to the reflectance limits used in determining the rate of darkening.

Table 3. Effect of treatments on pH decrease.

Treatment	pH Initial	pH 600 hr at 49°C	Rate (Δ pH/100 hr)	Per cent of control rate
Experiment 1				
Air [a]	6.05	4.74	0.177	100
Nitrogen [b]	6.05	4.88	0.131	74.0
Short heat	6.03	4.91	0.131	74.0
Long heat	5.30	4.80	0.192 [c]	108
Experiment 2				
Air	6.20	4.85	0.168	100
Carbon monoxide	6.20	4.80	0.161	95.9
Preservative	6.20	4.58	0.373	222

[a] Average of duplicates.
[b] Average of triplicates.
[c] This value is for the 5.3–5.0 pH range extrapolated to the 5.5–5.0 pH range.

It is clear that the primary reaction system responsible for the decrease in pH is nonoxidative and nonenzymic since neither inert atmospheres nor heat treatments prevent this change. The 26% decrease in rate of pH change exhibited by the nitrogen- and short-heat-treated dates suggests the presence of a secondary enzymic oxidative system. Since the carbon-monoxide- and long-heat-treated dates did not show similar significant rate reductions, proof of the existence of this secondary system must await further evidence. It is possible that oxygen and enzymes have only an indirect effect on the primary system. The marked effect of the preservative on the rate appears to be the result of methyl formate hydrolysis. Dates underwent a 0.5 pH unit decrease 24 hours after application of the preservative. Ethylene oxide, the other constituent of the preservative, had no influence on pH when tested alone. After the rapid initial change the rate of pH decrease of the preservative-treated dates was identical with that of the control dates.

A similar pattern of results is apparent between pH and reflectance, suggesting a relationship between the two. It is likely that the decrease in pH is a result of the same reaction systems that cause tissue

darkening. A decrease in pH during darkening has been observed in model browning systems (Hodge, 1953). The possibility that this change is the result of microbial activity is extremely remote, since the carbon-monoxide-, preservative-, and heat-treated dates all undergo pronounced decreases in pH.

Effect of treatments on sucrose inversion. The dates used in this work had a total sugar content of 82.9% on a dry-weight basis. Initially, 58.5% of the total sugar was sucrose, which after 600 hr at 49°C decreased to 17.6%. Plots of logarithm sucrose concentration against time produce straight lines, establishing the kinetics of the reaction as (pseudo-) first-order. The rate constants (k's) listed in Table 4 were calculated from the slopes and are given in units of reciprocal hours.

Table 4. Effect of treatments on sucrose inversion.

Treatment	Sucrose content (% dry weight)		Rate constant, $k \times 10^3$, hr^{-1}	Per cent of control rate
	Initial	600 hr at 49° C		
Air [a]	48.4	14.6	2.05	100
Nitrogen [b]	48.4	13.8	2.05	100
Short heat	47.7	44.4	0.104	5.6
Long heat	50.1	44.8	0.094	4.6
Experiment 2				
Air	49.3	14.4	1.94	100
Carbon monoxide	49.3	13.7	2.10	108
Preservative	49.3	13.0	2.22 [c]	114

[a] Average of duplicates.
[b] Average of triplicates.
[c] Estimated rate, not first-order.

The nature of the invertase of different varieties of dates and its role in ripening were reported by Vinson (1911). The data in Table 4 show that invertase is responsible for sucrose inversion in Deglet Noor dates during storage since inversion is strongly inhibited by the enzyme-inactivating heat treatments. The more rapid rates of inversion found for the carbon-monoxide- and preservative-treated dates are probably the result of their action on the tissue in causing a more rapid release of bound invertase. Many different chemicals are known to aid the release of invertase from cells (Neuberg and Mandl, 1950). The invertase of dates is much more heat-sensitive than phenolase, since it was destroyed by the short-heat treatment, which destroyed only 22% of the phenolase activity.

Effect of treatments on oxygen absorption and carbon dioxide production. Graphs of gas exchange data versus time gave straight lines during the major part of the experiment. The rates in Table 5, obtained from the slopes of these lines, are reported on the basis of dry date tissue, excluding the weight of the seeds except where seeds alone were studied.

Comparison of gas exchange rates in Table 5, Experiment 1, shows the presence of both enzymic and nonenzymic systems. A maximum of 36% of the oxygen and 29.8% of the carbon dioxide can be attributed to enzyme reactions. The production of carbon dioxide is almost completely inhibited in the absence of oxygen (Warburg experiment); thus it is produced mainly by reactions that require molecular oxygen. These results differ from those of Stadtman *et al.* (1946), who found that carbon dioxide production of blanched, sulfured apricots was only slightly retarded in nitrogen. The more rapid rate of gas exchange exhibited by the dates in the Warburg experiment was probably caused by the twofold greater amount of oxygen initially available. The gas exchange of date seeds is thought to be the result of respiration. Calculations show that a maximum of 21–22% of the gas exchange of intact dates can be attributed to seed respiration, assuming no diffusional limitations. The preservative (Experiment No. 2) had no effect on the rate of oxygen absorption. Since ethylene oxide, the active ingredient of the preservative, is an excellent sterilizing agent for dried fruits (Whelton *et al.*, 1946) it is apparent that the oxygen absorption observed is not a result of microbial respiration. This conclusion is supported by the low moisture content of the fruit and the absence of observable microbial growth or fermentation odor. The 29% slower carbon dioxide production by the preservative-treated dates might be caused by the lower pH of this fruit.

Table 5. Effect of treatments on oxygen absorption and carbon dioxide production.

Treatment	Oxygen absorption		Carbon dioxide production	
	Rate (μg/g/hr)	Per cent of control rate	Rate (μg/g/hr)	Per cent of control rate
Experiment 1				
Air [a]	0.867	100	1.23	100
Short heat	0.680	78.4	0.987	80.2
Long heat	0.555	64.0	0.864	70.2
Experiment 2				
Air	0.941	100	1.29	100
Preservative	0.941	100	0.915	71.0
Warburg				
Air (whole dates)	1.37	100	1.96	100
Nitrogen [a] (whole dates)			0.156	7.95
Air (seeds)	2.98 (.288)[b]	(21.0)[b]	3.48 (.431)[b]	(22.0)[b]

[a] Average of duplicates.
[b] Calculated for the seeds present in intact dates from the rate obtained with seeds alone and assuming no diffusional limitations.

At present, the reaction system or systems responsible for the major portion of gas exchange are unknown. They are apparently nonenzymic reactions. Enzymic reactions, which account for the minor portion of the oxygen and carbon dioxide, probably include enzymic browning, seed respiration, and tissue respiration.

Effect of treatments on enzyme activities. Peroxidase and phenolase activities were assayed after 27 and 602 hours at 49°C for the treatments of Experiment 2. The results (Table 6) indicate an increase in the apparent activity of phenolase and a decrease in that of peroxidase for all treatments. The increase in apparent activity of phenolase is probably caused by an increased extractability of the enzyme that resulted from tissue and cellular disorganization. Carbon monoxide and the preservative, both of which are cell poisons, caused higher phenolase activities 27 hours after treatment as well as after 602 hours. The phenolase of dates is reported to be a laccase (Rashid, 1950), so no inhibition by carbon monoxide would be expected. In all cases peroxidase activity decreased. Carbon monoxide caused a 33% decrease in activity 27 hours after treatment, whereas the preservative had little effect. Carbon monoxide is known to be an inhibitor of peroxidase.

Table 6. Effect of treatments on enzyme activities.

Treatment	Phenolase activity, (Δ absorbance/min)		Peroxidase activity, (Δ absorbance/min)	
	27 hr	602 hr	27 hr	602 hr
Air	0.031	0.085	0.090	0.041
Carbon monoxide	0.044	0.161	0.060	0.055
Preservative	0.071	0.091	0.083	0.053

These results suggest that native date phenolase is associated with an insoluble tissue fraction. The association is apparently disrupted by deterioration of the tissue and by treatment with certain chemicals. Biedermann's (1956) observation that tyrosinase is strongly linked with the solid particles of apple and pear pulp agrees with this interpretation. In addition, Mayer and Friend (1960), working with sugar beets, obtained evidence of phenolase binding to chloroplasts or some structure therein. It appears that peroxidase occurs in the free or soluble state in dates.

PRACTICAL SIGNIFICANCE OF RESULTS

The darkening of domestic dates is caused by both oxidative and nonoxidative browning systems. Therefore, retardation of darkening necessitates the inhibition of either or both of these systems. The work reported shows that oxidative browning can be inhibited by holding dates in inert gas

atmospheres. Although inhibition of oxidative browning retards over-all darkening by only 10–20% at the high storage temperature (49°C) used in this work, other studies, not yet reported, show that over-all darkening is slowed 40–50% at lower temperatures (28.4°C). The use of inert gas atmospheres requires a gas-impermeable package for successful inhibition of oxidative browning. This might also be supplemented by in-package oxygen scavenging systems such as glucose oxidase-catalase.

Nonoxidative browning is not affected by inert gas atmospheres. There was an indication that over-all darkening was retarded somewhat by lowering the pH of the date tissue. Further work is needed to assess the value of such a treatment and to attempt to retard nonoxidative browning by other means.

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RESEARCH NOTES

November 15, 1960

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A possible error was pointed out to us in the paper published in the July-August *Food Research*, "A Study of a Spectrophotometric Method for the Flavor Evaluation of Insecticide Treated Vegetables" by Daniel Rosenfield, Arnold I. Epstein, Elizabeth F. Stier, and Walter A. Maclinn.

Dr. Galvin called to tell us that the infrared curves shown for the insecticide treated samples are almost identical to those for Dow Corning stopcock grease in carbon disulfide. They had had this particular curve occur many times and had finally traced it to the stopcock grease used in separatory funnels.

Dr. Galvin was kind enough to send us a curve of this silicone stopcock grease in carbon disulfide, which almost matches the curve in the paper except the absorption is weaker at 12.5 mμ.

After having checked the literature on infrared curves for the insecticides, we have found none which match exactly those curves shown in the paper. However, the pure insecticides do have peaks in the area shown in our curves. We did not expect that the curves from the vegetable extracts would necessarily be like those of the pure insecticide, since it has been indicated that the "off-flavor" which occurs in some insecticide treated vegetables could be caused by breakdown products of the insecticide and not the pure insecticide. It has been shown that some insecticides enter into normal plant metabolism and are not detectable as insecticides in the plant.

The only thing which puzzles us is why the untreated samples which were handled identically to the treated samples in the extraction procedure apparently were never contaminated by the stopcock grease. We do not know the answer to the question and can only surmise that insecticides could produce a spectrum identical to stopcock grease. Additional evidence on this point is that in no case where insecticide samples were found to have "no flavor difference" in panel evaluations, were spectral curves obtained which were different from untreated samples.

In additional correspondence with the Beckman Instrument Company the similarity between the curves for silicones and the insecticides was also noted. However, they concluded that the absorption must be connected to the pesticide in some manner, since both treated and untreated samples were handled alike during preparation for spectrophotometric work.

Sincerely,
ELIZABETH F. STIER
Assistant Research Specialist

The search for Truth is in one way hard and in another easy. For it is evident that no one can master it fully nor miss it wholly. But each adds a little to our knowledge of Nature, and from all the facts assembled there arises a certain grandeur.

—ARISTOTLE